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(54) Title: A LEPTIN BINDING PROTEIN AND ITS USE IN METHODS FOR DIAGNOSING AND TREATING ABNORMALITIES OF THE ENDOGENOUS LEPTIN PATHWAY

(57) Abstract

The present invention discloses a leptin binding protein that plays a novel role in the regulation of adiposity and fat content of animals, particularly in mammals. The leptin binding protein binds to leptin *in vivo*, forming a leptin-leptin binding protein complex that functions to provide a means for regulating body weight and adiposity. The present invention exploits this novel role of the leptin binding protein by providing means for diagnosing, monitoring, treating and curing abnormalities of the endogenous leptin pathway. Apolipoprotein J serves as an example of this new class of leptin binding protein.

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A LEPTIN BINDING PROTEIN AND ITS USE IN METHODS FOR DIAGNOSING AND TREATING ABNORMALITIES OF THE ENDOGENOUS LEPTIN PATHWAY

GOVERNMENTAL SUPPORT

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FIELD OF THE INVENTION

The present invention relates to methods and compositions for modulation of body mass. In particular, a leptin binding protein has been identified. The concentration of leptin bound to the leptin binding protein provides an indication of the biological activity of leptin. Increasing the concentration of the leptin binding protein leads to an increase leptin activity, a decrease in body mass and more importantly, a decrease in levels of fat. The invention includes important diagnostic and therapeutic implications in homeostasis of body weight and fat tissue mass.

BACKGROUND OF THE INVENTION

Leptin and Genetic Control of Body Mass

Obesity, defined as an excess of body fat relative to lean body mass, is associated with important psychological and medical morbidities, the latter including hypertension, elevated blood lipids, and Type II or non-insulin-dependent diabetes melitis (NIDDM) and decreased life expectancy. There are 6-10 million individuals with NIDDM in the U.S., including 18% of the population of 65 years of age [Harris *et al.*, *Int. J. Obes.*, 11:275-283 (1987)]. Approximately 45% of males and 70% of females with NIDDM are obese, and their diabetes is substantially improved or eliminated by weight reduction [Harris, *Diabetes Care*, **14(3)**:639-648 (1991)]. Both obesity and NIDDM are strongly heritable, though few of the predisposing genes have been identified. The

molecular genetic basis of these metabolically related disorders is an important, poorly understood problem.

The assimilation, storage, and utilization of nutrient energy constitute a complex homeostatic system central to survival of metazoa. Among land-dwelling mammals, storage in adipose tissue of large quantities of metabolic fuel as triglycerides is crucial for surviving periods of food deprivation. The need to maintain a fixed level of energy stores without continual alterations in the size and shape of the organism requires the achievement of a balance between energy intake and expenditure. However, the molecular mechanisms that regulate energy balance remain to be elucidated. The isolation of molecules that transduce nutritional information and control energy balance will be critical to an understanding of the regulation of body weight in health and disease.

Examination of the concordance rates of body weight and adiposity amongst mono- and dizygous twins or adoptees and their biological parents have suggested that the heritability of obesity (0.4-0.8) exceeds that of many other traits commonly thought to have a substantial genetic component, such as schizophrenia, alcoholism, and atherosclerosis [Stunkard *et al.*, *N. Engl. J. Med.*, 322:1483-1487 (1990)]. Familial similarities in rates of energy expenditure have also been reported [Bogardus *et al.*, *Diabetes*, 35:1-5 (1986)]. Genetic analysis in geographically delimited populations has suggested that a relatively small number of genes may account for the 30-50% of variance in body composition [Moll *et al.*, *Am. J. Hum. Genet.*, 49:1243-1255 (1991)].

The presence and degree of obesity are usually determined by reference to the absolute weight of an individual when compared to age and height matched ideals, or by reference to the individual's body mass index, that is, body weight (in kilograms) divided by height (in meters^2), compared with age matched ranges.

A major advance in understanding the molecular basis for obesity occurred with the cloning of the *ob* gene. The mouse obesity (*ob*) gene encodes an adipose tissue-derived signaling factor for body weight homeostasis [Zhang *et al.*, *Nature*, **372**:425 (1994); U.S. Patent Application No. 08/292,345 filed August 17, 1994; U.S. Patent Application No. 08/483,211, filed June 7, 1995, each of which is hereby incorporated by reference in its entirety]. Several recent studies have shown that recombinant OB protein (leptin) purified from *Escherichia coli* can correct the obesity related phenotypes in *ob/ob* mice when exogenously administered [Campfield *et al.*, *Science*, **269**:546 (1995); Pellymounter *et al.*, *Science*, **269**:540, (1995); Halaas *et al.*, *Science*, **269**:543 (1995); Stephens *et al.*, *Nature*, **377**:530 (1995)]. Weight-reducing effects of recombinant leptin were also observed in normal mice and mice with diet-induced obesity. Although the target tissues that mediate the effects of leptin have not yet been defined, the instant inventors have predicted the brain as a target of leptin activity. Indeed, the work of Campfield *et al.* (*supra*) and Stephens *et al.* (*supra*) demonstrates that leptin introduced into the lateral or third brain ventricle is effective at low doses, arguing for a direct central affect of the leptin molecule.

Recent studies have suggested that obese humans and rodents (other than *ob/ob* mice) are not defective in their ability to produce leptin mRNA or protein and generally produce higher levels than lean individuals [Maffei *et al.*, *Nature Med.*, **1**:1155 (1995); Considine *et al.*, *J. Clin. Invest.*, **95**:2986 (1995); Lonnqvist *et al.*, *Nature Med.*, **1**:950 (1995); Hamilton *et al.*, *Nature Med.*, **1**:953 (1995)]. These data suggest that resistance to normal or elevated levels of leptin may be important factors in human obesity. However, a recent report of identification of a leptin receptor did not identify any mutations in the *ob* allele [Tartaglia *et al.*, *Cell*, **83**:1263-1271 (1995)].

Apolipoprotein J defines a heterogeneous subclass of human plasma high density proteins (HDL) having a bimodal distribution of molecular mass 70-90 kD and 200 kD or larger. Apolipoprotein J as a unique 70 kD component of high density

lipoproteins in human plasma consists of two disulfide linked subunits designated ApoJ α, which is between 34 and 36 kD and ApoJ ß, which is between 36 and 39 kD.

A comparison of the peptide map suggests that ApoJ α and ApoJ ß are not identical but share limited regions of homology. It appears that the ApoJ α and ß subunits are derived by proteolytic cleavage of a common precursor [de Silva *et al.*, **265**:14292-14297 (1990)]. In blood, there are two sources of apolipoprotein J: high density lipoproteins and platelets. In circulating apolipoprotein J, 30-40% appear to be sequestered within platelets. High levels ApoJ gene expression occurs in megakaryocytes, which are the precursors of blood platelets, and platelet apolipoprotein J is stored in alpha granules and released by platelet activation. There is a positive correlation between plasma apolipoprotein J levels and total cholesterol triglyceride. Low density lipoprotein cholesterol apoB and apoE may reflect the extent of platelet activation at sites a vascular injury. The HDL particles containing apolipoprotein J comprise unique and previously undescribed subclasses.

It has not been unequivocally established that apolipoprotein J is, in fact, an apolipoprotein. The available evidence does favor this clarification. On the basis of gene structure and sequences of potential amphipathic helices, apolipoprotein J is clearly not a member of the apolipoprotein gene family including the apoA's, apoC's and apoE's. However, when evaluated in plasma and zero spinal fluid, apolipoprotein J is associated with lipid. The protein is secreted by culture hepatocytes as a lipoprotein enriched in triglyceride compared to the plasma form. The best evidence for the finding that apolipoprotein J is a lipoprotein is that ApoJ is recognized as a binding partner by gp330, a member of the LDL receptor gene family. Internalization and degradation of apolipoprotein J by cultured F9 cells is mediated by gp330.

Earlier reports have shown that apolipoprotein J has been found to transport proteins. One such example is that it transports apoAI in human plasma [Jenne *et al.*, *J. of Biol. Chem.* **266**:11030-11036 (1991)]. Apolipoprotein J is highly conserved across

species. In addition to lipid transport and regulation of complement function, it seems to be involved in sperm maturation, programmed cell death and membrane recycling. The primary responsibility of ApoJ has been attributed to the maintenance of cell integrity through participation in the process of membrane surveillance, stabilization, remodeling and repair. The cytoprotective function of apolipoprotein J is particularly important to the epithelial cells of organs exposed to a multitude of potentially damaging agents present in aqueous environments [Jordan-Starck *et al.*, *Current Opinion in Lipidology* 3:75-85 (1992)].

Therefore, there is a current need for methods and related compositions that could be used in detecting physiological obesity or other conditions related to abnormalities of the endogenous leptin pathway. Furthermore, the use of such a method and composition in the treatment of physiological obesity or other conditions related to abnormalities of the endogenous leptin pathway, could potentially remedy these heretofore untreatable disorders.

The citation of any reference herein should not be deemed as an admission that such reference is available as prior art to the instant invention.

SUMMARY OF THE INVENTION

According to the present invention a leptin binding protein plays a role in the regulation of adiposity and fat content of animals, particularly in mammals. This role involves the interaction between the leptin binding protein and the product of the *OB* gene, leptin. *In vivo*, leptin and the leptin binding protein associate forming a "L-LBP" complex, which apparently functions to provide a means for regulating body weight and adiposity. The present invention exploits the novel role of the leptin binding protein by providing means for diagnosing, monitoring, treating and curing abnormalities of this regulatory process in mammals.

The leptin binding protein has a binding affinity for leptin and co-purifies with leptin when leptin is purified on a leptin affinity column. The leptin binding protein has an apparent molecular weight of about 80 kD determined by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions and an apparent molecular weight of about 40 kD determined by SDS-PAGE under reducing conditions. The leptin binding protein binds to an ApoJ-specific antibody in ELISA and Western analysis. In preferred embodiments the N-terminal amino acid sequence of at least one of the monomeric subunits of the 80 kD dimer is SEQ ID NO:1

XQTVSDNELQEMSNQGSKYVNKEI

where X is an aspartic acid, a modified aspartic acid or hydrogen (*i.e.*, no amino acid).

In specific embodiments of the present invention, apolipoprotein J is a leptin binding protein involved in the regulation of adiposity and fat content of animals. Accordingly, leptin and apolipoprotein J associate forming a L-LBP complex specifically termed herein a "L-aJ" complex. Thus, apolipoprotein J serves as an example of this new class of leptin binding protein.

The present invention provides a leptin binding protein with the following characteristics: (a) it co-purifies with leptin when leptin is purified from murine or human serum by a leptin affinity column; (b) it has a binding affinity for leptin; and (c) it has a molecular weight of about 40 kD as determined by SDS-PAGE under reducing conditions. In its non-reduced form, the leptin binding protein has a molecular weight of about 80 kD determined by SDS-PAGE under non-reducing conditions. Leptin binding protein binds an antibody specific for ApoJ in ELISA and Western analysis. In a specific embodiment the leptin binding protein is apolipoprotein J. In a more specific embodiment, the apolipoprotein J is a particular subclass of apolipoprotein J.

The present invention is directed to compositions useful for either diagnosing or for treating abnormalities in the endogenous leptin pathway, such as physiological obesity. This aspect of the present invention includes the use of the leptin binding protein either alone or in conjunction with leptin in compositions for use in medically assisting mammalian subjects who have abnormalities in the regulatory control of their body weight. In all of the embodiments of this aspect of the invention the leptin binding protein can be apolipoprotein J. In one embodiment, the composition consists essentially of purified leptin binding protein and leptin. In another embodiment the composition comprises a purified form of leptin bound to the leptin binding protein. In still another embodiment, a composition comprises a purified formulation of the leptin binding protein and leptin. In preferred embodiments, the ratio of leptin to the leptin binding protein is approximately 1:1. The leptin binding protein is reversibly associated with at least some of the leptin. In preferred embodiments, approximately half of the leptin is bound to the leptin binding protein. In the most preferred embodiments, at least about 75% of the leptin present is bound to the leptin binding protein.

The leptin can be a mammalian form of leptin. In preferred embodiments, the leptin is a murine form of leptin. In more preferred embodiments, the leptin is the human form of leptin.

The leptin binding protein can be a mammalian form of the leptin binding protein. In preferred embodiments, the leptin binding protein is a murine form of the leptin binding protein. In more preferred embodiments, the leptin binding protein is the human form of the leptin binding protein.

All of the forms of leptin and the leptin binding protein in the present invention can be derived from either their natural source, a recombinant source or by chemical synthesis.

The present invention also includes antibodies to the L-LBP complex and antibodies to the leptin binding protein. For this aspect of the invention apolipoprotein J may be the leptin binding protein and the antibody to the leptin binding protein is specific for apolipoprotein J. Similarly, in a specific embodiment, an antibody to the L-LBP complex is specific for the L-aJ complex.

Preferably, an antibody of the invention is specific for an epitope that is either created or made accessible by the association of leptin with the leptin binding protein. Such an antibody may still cross-react with either leptin or the leptin binding protein, *e.g.*, apolipoprotein J. However, in a preferred embodiment, such cross-reactivity is minimal, and occurs with an affinity constant well below that for specific antibody-antigen binding. In particular embodiments, an antibody specific for an epitope so formed will have an association constant for either free leptin or free leptin binding protein that is below 10^8M^{-1}, preferably below 10^7M^{-1} and most preferably below 10^6M^{-1}.

The antibodies of the present invention may be polyclonal antibodies, or monoclonal antibodies, or chimeric (bispecific) antibodies and all such variants are considered to be included herein. Derivatives of antibodies and fragments that retain antigen binding sites are also included in the present invention. Such derivatives include antibodies modified so as to possess a phosphorylation site, a reactive sulfhydryl or other such biochemical modification. All of the antibodies can further comprise a detectable label as described below.

A related aspect of the present invention includes an immortal cell line that produces a monoclonal antibody against the L-LBP complex. One such immortal cell line produces a monoclonal antibody against leptin bound to apolipoprotein J, the "L-aJ" complex.

Another aspect of the invention includes pharmaceutical preparations for treating subjects suffering from an abnormality in their endogenous leptin pathway. In some of the embodiments of this aspect of the invention the abnormality in the endogenous leptin pathway results in decreased leptin activity. In one embodiment, there is provided a pharmaceutical preparation for the treatment of abnormalities in the endogenous leptin pathway consisting essentially of the leptin binding protein and a pharmaceutically acceptable carrier. Still another embodiment of this aspect of the invention includes a pharmaceutical preparation comprising purified leptin binding protein and a pharmaceutically acceptable carrier. In some embodiments, the latter pharmaceutical preparation also contains leptin. Either leptin or the leptin binding protein, or both, may be obtained from recombinant sources, or even prepared by peptide synthesis.

This aspect of the present invention also includes methods of making the pharmaceutical preparations for treating subjects suffering from an abnormality in their endogenous leptin pathway. In some embodiments of this aspect of the invention the pharmaceutical preparations are made by adding a suitable amount of the leptin binding protein to a pharmaceutically acceptable carrier. In preferred embodiments, the leptin binding protein is purified leptin binding protein. In some embodiments, a suitable amount of leptin is also added to the mixture. The order of additions in this aspect of the invention is not important. In preferred embodiments both the leptin and the leptin binding protein are purified prior to mixing. In still other embodiments the leptin binding protein, purified leptin, and a pharmaceutically acceptable carrier are added together to form the pharmaceutical composition.

In some embodiments of this aspect of the invention both the leptin and the leptin binding protein are mammalian in origin. In a specific embodiment both the leptin and the leptin binding protein are murine in origin. In a preferred embodiment, either the leptin or the leptin binding protein are human. More preferably both are human.

Either the leptin or the leptin binding protein may be derived from a recombinant source.

In addition, the present invention includes methods for isolating the L-LBP complex produced by genetic engineering, from bacterial, insect, or animal cells. In a specific embodiment the L-LBP complex is a L-aJ complex. In one embodiment, the method of isolating the complex uses a leptin affinity column.

The invention also includes a pharmaceutical preparation comprising a pharmaceutically acceptable carrier and an antagonist of the leptin binding protein. In another embodiment a pharmaceutical composition comprises a pharmaceutically acceptable carrier and an antagonist to the L-LBP complex. Such embodiments may serve to inhibit the effect of the L-LBP complex and be useful in the treating diseases where patients experience an unhealthy to life threatening amount of weight such as in anorexias, certain cancers, and AIDS.

An antagonist of the leptin binding protein can be an antibody that binds to and neutralizes the activity of the leptin binding protein, or a fragment of the leptin binding protein that binds to leptin but does not serve as a mediator in the binding of leptin to the leptin receptor, or a small molecule antagonist of the leptin binding protein. An antagonist for the L-LBP complex can be an antibody that binds to and neutralizes the activity of the L-LBP complex. This antibody would preferably not cross-react with free leptin. Alternatively the antagonist for the L-LBP complex is a small molecule. In specific embodiments of this aspect of the invention, the leptin binding protein is apolipoprotein J.

Yet another aspect of the present invention includes expression vectors containing nucleic acids encoding the leptin binding protein, or the leptin binding protein and leptin. In one embodiment, two expression vectors are used, so as to allow both the leptin binding protein and leptin to be expressed. In another embodiment, an

expression vector contains a nucleic acid that encodes both the leptin binding protein and leptin. These coding sequences can be operatively linked to an expression control sequence.

One or more vectors of the present invention may be homologously recombined in a chromosome in a cell. In some embodiments of this aspect of the invention, the cell contains a nucleic acid that has been disrupted, and the cell is unable to express a functional form of the leptin binding protein. In one specific example of this aspect of the invention, the cell can be a chicken DT40 cell. In other embodiments, the cell contains a nucleic acid encoding the leptin binding protein that has been disrupted, and in addition, either lacks leptin or has a non-functional *ob* gene, such that the cell is neither able to express a functional form of the leptin binding protein or a functional form of leptin.

In other preferred embodiments of this aspect of the invention, the cell is mammalian and can be placed into a mammalian blastocyst. The blastocyst can then be re-implanted into a pseudopregnant female mammal to generate a transgenic animal. In one embodiment of this aspect of the invention, the transgenic animal contains a nucleic acid that has been disrupted, and the transgenic animal cannot express a functional form of the leptin binding protein. One use for such an animal model would be as a research tool for identifying treatments that can help regulate body weight in the absence of the leptin binding protein. In another embodiment the transgenic animal cannot express a functional form of the leptin binding protein or a functional form of leptin. Such an animal would be useful to distinguish specific drugs that mimic the leptin binding protein role in the endogenous leptin pathway from drugs that may mimic the leptin binding protein in other ways which otherwise could lead to a false positive. This is particularly relevant when apolipoprotein J is the leptin binding protein since apolipoprotein J plays so many roles in the body's metabolism. In preferred embodiments the transgenic animal is a mouse that can be used as an animal model.

When the transgenic mouse contains a disrupted nucleic acid as described above, the transgenic animal is a "knockout" mouse. For all of the embodiments of this aspect of the invention, apolipoprotein J can be the leptin binding protein.

An alternative aspect of the present invention includes antisense nucleic acid that functions by hybridizing to the mRNA encoding the leptin binding protein. In some embodiments the antisense nucleic acid is RNA. In other embodiments the antisense nucleic acid is DNA. In either case, preferably the antisense nucleic acid is a synthetic RNA or DNA containing non-naturally occurring phosphoester-analog bonds. The present invention also includes a recombinant DNA vector having a DNA sequence which, on transcription, produces an antisense ribonucleic acid against an mRNA coding for the leptin binding protein. For all of the embodiments of this aspect of the invention, apolipoprotein J can be the leptin binding protein.

In still another aspect of the present invention there is a ribozyme that catalyzes the cleaving of a precursor RNA as part of the process of converting a precursor RNA to a either a functional or a non-functional mRNA transcript that encodes the leptin binding protein. The precursor RNA contains the exons that encode the leptin binding protein, and one or more introns that have one or more sites for cleaving the introns from the precursor RNA. Alternatively, the ribozyme may cleave the mRNA transcript that encodes the leptin binding protein and thereby inactivates it. In one embodiment the ribozyme is a *Tetrahymena*-type ribozyme. In another embodiment the ribozyme is a Hammerhead-type ribozyme. The present invention also includes a recombinant DNA molecule which, upon transcription, produces the ribozyme. For all of the embodiments of this aspect of the invention, apolipoprotein J can be the leptin binding protein.

Another aspect of the present invention includes methods of making the compositions and pharmaceutical preparations of the present invention. In specific embodiments of the invention, the leptin binding protein is apolipoprotein J.

In the broadest aspect, a composition of the leptin binding protein and leptin can be prepared by admixing a composition comprising leptin with a composition comprising leptin binding protein. In a preferred embodiment, purified leptin or purified leptin binding protein or both are included in the admixture. In a more preferred embodiment, both reagents are purified and form a purified formulation. Any bound leptin or leptin binding protein can be isolated from any other components in the admixture.

The present invention further provides the use of methods for detecting either the leptin binding protein or the L-LBP complex in mammals as a means for diagnosing abnormalities or for monitoring the progress of the treatment. In specific embodiments of the methods of the present invention, the leptin binding protein is apolipoprotein J; in such embodiments the L-LBP complex can be a L-aJ complex.

In one embodiment of this aspect of present invention the method comprises the steps of: (a) contacting a biological sample with a detectable binding partner that has an affinity for leptin bound to the leptin binding protein (the "L-LBP" complex), under conditions that allow the detectable binding partner to associate with the L-LBP complex when the L-LBP complex is present in the biological sample; and (b) detecting the detectable binding partner associated with the L-LBP complex, wherein detecting the detectable binding partner associated with the (L-LBP) complex is correlated to detecting leptin bound to the leptin binding protein in the biological sample. In preferred embodiments of this aspect of the invention, the detectable binding partner is an antibody raised against the (L-LBP) complex that contains a fluorophore that has an emission spectrum that is red-shifted when the detectable binding partner associates with the (L-LBP) complex. Various assay techniques, including but not limited to sandwich, direct binding, inhibition assays in which either the specific binding partner or control antigen is labelled, displacement, release, diffusion, and precipitation can be employed to practice this aspect of the invention.

Such assays may be preferred in homogeneous (all reagents in solution or suspension) or heterogeneous (reagents on a solid phase and others in solution or suspension) formats. For example, displacement, precipitation, and inhibition assays can be performed in a homogeneous format. Displacement, inhibition, sandwich, and direct binding assays can be performed in a heterogenous format. Examples of heterogeneous format assays include ELISA, immunochromatographic assays, and biosensor or optical sensor-type assays.

In some embodiments of this aspect of the invention, a method of detecting leptin bound to the leptin binding protein in a biological sample comprises the steps of: (i) contacting a biological sample with a binding partner attached to a solid support, wherein the "attached binding partner" has an affinity for leptin bound to apolipoprotein, under conditions that allow the attached binding partner (or first binding partner) to associate with the L-LBP complex when the complex is present in the biological sample and thereby forms an attached binding partner-L-LBP complex or "solid support complex"; (ii) contacting the solid support complex with a detectable binding partner (or second binding partner), that has an affinity for the solid support complex, so that when the solid support complex is present, the detectable binding partner associates with it; (iii) detecting the detectable binding partner associated with the solid support complex, wherein detecting the detectable binding partner associated with the solid support complex can be correlated to detecting leptin bound to the leptin binding protein in a biological sample. If necessary, a washing step can be performed.

A suitable biological sample for the present invention, includes an animal tissue sample, an animal body fluid, an animal excretory material, and an animal secretory material. In a preferred embodiment the biological sample is chosen from blood, serum, plasma, urine, and cerebral spinal fluid. However, any appropriate biological sample is acceptable as will be understood by those with skill in the art with reference to the disclosure herein.

This aspect of the present invention also includes methods of quantifying the L-LBP complex in a biological sample. Embodiments of this aspect of the invention can use any of the various means of detecting a detectable binding partner associated with the L-LBP complex, and includes the methods of detecting the L-LBP complex described above. In these embodiments a further step of quantifying the associated detectable binding partner is performed and then correlated to the quantity of leptin bound to the leptin binding protein in the biological sample. Such means of quantifying are well known in the art.

A particular advantage of the present invention is the recognition that the association of leptin with the leptin binding protein may have adversely affected the accuracy of assays for leptin levels in normal *versus* anorexic individuals. Assays, such as the sandwich ELISA assays, may under-estimate the leptin levels in the normal group, where much of the leptin is associated with the leptin binding protein. In contrast, in the obese group, most of the leptin is not bound to the leptin binding protein. A further advantage of the instant invention is that it provides for detecting and quantifying both free and associated leptin, thus allowing for a more accurate determination of leptin levels in an individual.

The specific binding partners used for these methods include but are not limited to an anti-leptin antibody, solubilized leptin receptor and leptin-binding derivatives and fragments thereof, an anti-leptin binding protein antibody, solubilized leptin binding protein receptor and LBP binding fragments and derivatives thereof, an anti-L-LBP complex antibody, an antigen binding fragment thereof, derivatives thereof and combinations thereof. In a specific embodiment, apolipoprotein J is the leptin binding protein and an anti-apolipoprotein J antibody, a solubilized apolipoprotein J receptor, an anti-L-aJ complex antibody, an antigen binding fragment thereof, derivatives thereof and combinations thereof are also included.

Another aspect of the present invention includes methods for diagnosing an abnormality in the endogenous leptin pathway in a mammalian subject. An embodiment of this method comprises the steps of: (a) determining the amount of one or more forms of the leptin binding protein in a given biological sample acquired from the mammalian subject; and (b) comparing that amount to a range of determined amounts of the corresponding form or forms of the leptin binding protein in mammals having a normal endogenous leptin pathway. A determined amount from step (a) greater than or less than the range of amounts indicates a diagnosis of an abnormality in the endogenous leptin pathway.

In one embodiment of this aspect of the invention the form of the leptin binding protein determined is the total leptin binding protein, *i.e.*, all of the leptin binding protein present in the sample regardless of whether it is free or associated with another protein, *e.g.*, leptin. In another embodiment the form of the leptin binding protein determined is restricted to that bound to leptin. In still another embodiment the form of the leptin binding protein determined is restricted to that not bound to leptin. In additional embodiments the form of the leptin binding protein can be the leptin binding protein bound to an alternative protein molecule, or particle (such as an HDL particle). Still, in other embodiments, the form of the leptin binding protein determined is restricted to free leptin binding protein, *i.e.*, a form that is not bound to any other protein or particle. Free leptin binding protein, as used herein, includes, however, monomers, dimers and other higher oligomeric forms of the leptin binding protein. In addition, combinations (such as monomers *plus* dimers) and differences (such as total leptin binding protein *minus* free leptin binding protein) are forms envisioned by the present invention.

Furthermore, this aspect of the present invention includes further analysis of the determined amounts of these forms of the leptin binding protein. One embodiment includes an additional step (c) of calculating a quantitative relationship between the amount of the form of the leptin binding protein determined in the biological sample,

and the amount of one or more other forms of the leptin binding protein determined in that biological sample, wherein a relative value for the biological sample is determined; and an additional step of (d) comparing the relative value of step (c) to a range of the corresponding relative values determined in mammals having a normal endogenous leptin pathway, wherein a relative value from step (c) greater than or less than the range of the relative values in mammals having a normal endogenous leptin pathway indicates a diagnosis of an abnormality in the endogenous leptin pathway.

The mammals having a normal endogenous leptin pathway for this aspect of the present invention are generally from the same species as the corresponding mammalian subject, although in certain cases closely related species may be appropriate. Further, as used herein, the term "greater than or less than the range of amounts" and the term "lesser or greater than the range of values" indicate that a determined amount for the mammalian subject is outside of the statistically determined range of the amounts/values for mammals having a normal endogenous leptin pathway. A skilled artisan in the field, armed with the methods disclosed herein, would know how to calculate the appropriate statistical value, in order to make the correct determination.

A variation of an embodiment for diagnosing an abnormality in the endogenous leptin pathway as described above, pertains specifically to the diagnosis of physiological obesity. In this variation, the determined amount of one or more forms of the leptin binding protein in a given biological sample acquired from the mammalian subject that is less than the range of amounts for mammals having a normal endogenous leptin pathway is indicative of a diagnosis of physiological obesity. In this specific variation of the above embodiment, the form of the leptin binding protein determined can be total leptin binding protein. (In this regard, when the leptin binding protein is apolipoprotein J, the relevant form of total apolipoprotein J may be the total amount of a sub-class of apolipoprotein J.) Leptin binding protein bound to leptin is also a suitable form of leptin binding protein for this analysis. In addition, calculated or

determined combinations or differences of these states of leptin binding protein are also of value.

Another specific embodiment of this aspect of the invention describes a method for diagnosing an abnormality in the endogenous leptin pathway in a mammalian subject comprising the steps of: (a) determining the amount of leptin bound to the leptin binding protein in a biological sample acquired from the mammalian subject; and (b) comparing the determined amount from step (a) to a range of amounts of leptin bound to the leptin binding protein determined in mammals having a normal endogenous leptin pathway; wherein a determined amount from step (a) greater than or less than the range of amounts indicates a diagnosis of an abnormality in the endogenous leptin pathway.

A variation of this specific embodiment includes the additional step of (c) calculating a quantitative relationship between the amount of leptin bound to the leptin binding protein in the biological sample determined in step (a) above, and the amount of one or more other forms of leptin in the biological sample, wherein a relative value for the biological sample is determined; and the additional step of (d) comparing the relative value of step (c) to a range of the relative values determined in mammals having a normal endogenous leptin pathway, wherein a relative value from step (c) greater than or less than the range of the relative values in mammals having a normal endogenous leptin pathway indicates a diagnosis of an abnormality in the endogenous leptin pathway. Either the amount of total leptin, free leptin (not bound to the leptin binding protein), or both, are suitable forms of leptin to compare the amount of leptin bound to the leptin binding protein.

An alternative embodiment is a method for diagnosing an abnormality in the endogenous leptin pathway in a mammalian subject comprising the additional steps of (c) determining the amount of leptin bound to the leptin binding protein in a mammalian tissue sample containing cells that have the leptin receptor; (d) comparing

the determined amount from step (c) to a range of amounts of leptin bound to the leptin binding protein determined in mammals having a normal endogenous leptin pathway; (e) calculating a quantitative relationship between the amount of leptin bound to the leptin binding protein in the tissue sample, and the amount of leptin-receptor in the tissue sample, wherein a relative value for the tissue sample is determined; and (f) comparing the relative value of step (e) to a range of the relative values determined in mammals having a normal endogenous leptin pathway, wherein a relative value from step (e) greater than or less than the range of the relative values determined in mammals having a normal endogenous leptin pathway indicates a diagnosis of an abnormality in the endogenous leptin pathway.

According to another aspect of the present invention, there is provided a method of diagnosing abnormalities in the endogenous leptin pathway in a mammalian subject comprising the steps of, first, determining at least one parameter in a biological sample acquired from the mammalian subject selected from the group consisting of 1) an absolute amount of leptin bound to the leptin binding protein, 2) an absolute amount of leptin not bound to the leptin binding protein, 3) a percent of total leptin that is bound to the leptin binding protein, 4) a percent of total leptin that is not bound to the leptin binding protein, 5) the ratio of (an absolute amount of leptin that is bound to the leptin binding protein) to (an absolute amount of total leptin), 6) the ratio of (an absolute amount of leptin that is bound to the leptin binding protein) to (an absolute amount of leptin that is not bound to the leptin binding protein), and 7) the ratio of (an absolute amount of leptin that is not bound to the leptin binding protein) to (an absolute amount of total leptin). Next, the determined parameter is compared with a range of values for the parameter in the same type of mammal without abnormalities in the endogenous leptin pathway. A determined amount lesser or greater than the range of values indicates a diagnosis of an abnormality in the endogenous leptin pathway. As indicated earlier the mammalian subject in the determining step can be a human.

In each of the foregoing embodiments, deviations from the range of normal values indicates an abnormality in the endogenous leptin pathway. Decreased values for leptin bound to the leptin binding protein as compared to normal amounts, whether as an absolute value, as a percentage of the total leptin, or in a ratio with the total leptin concentration suggests insufficient activity in the endogenous leptin pathway, *e.g.*, favoring obesity, whereas the corresponding increase of these determinations compared to normal amounts suggests hyperactivity of the endogenous leptin pathway, *e.g.*, favoring anorexia.

The present invention also includes methods for treating abnormalities in the endogenous leptin pathway in a mammalian subject. One embodiment comprises the step of administering a composition comprising purified leptin binding protein. An alternative embodiment comprises the step of administering a composition consisting essentially of leptin associated with the leptin binding protein. According to another embodiment of the present invention, there is provided a method of treating abnormalities in the endogenous leptin pathway in a mammalian subject comprising the step of administering at least one treatment dose consisting essentially of the leptin binding protein and a pharmaceutically acceptable carrier. Yet another embodiment comprises the step of administering a composition comprising a purified formulation of the leptin binding protein and leptin. A preferred embodiment comprises the step of administering a composition comprised of a purified formulation of the leptin binding protein and human leptin. In specific embodiments of the foregoing, the leptin binding protein is apolipoprotein J.

A related embodiment of a method for treating abnormalities in the endogenous leptin pathway in a mammalian subject comprises the step of administering at least one dose of a pharmaceutical preparation comprising purified leptin binding protein and a pharmaceutically acceptable carrier. Another related embodiment comprises the step of administering a pharmaceutical preparation comprising an admixture of the leptin binding protein associated with leptin and a pharmaceutically acceptable carrier. As

stated above, the leptin binding protein can be apo J. In a specific embodiment, leptin is recombinant leptin. Preferably, both apo J and leptin are human xenoforms of the protein.

According to yet another aspect of the present invention, there is provided a method of treating abnormalities in the endogenous leptin pathway in a mammalian subject comprising the step of administering to the mammalian subject at least one treatment dose of a composition according to the present invention. This aspect of the present invention for treating abnormalities in the endogenous leptin pathway in a mammalian subject includes the step of administering at least one dose of a composition comprising a purified formulation of leptin and a leptin binding protein. In a preferred embodiment, the composition contains mammalian leptin. In a more preferred embodiment the leptin is human leptin. In another embodiment the leptin is derived from a recombinant source.

This aspect of the invention also includes the step of administering at least one dose of a pharmaceutical preparation comprising purified apolipoprotein J and a pharmaceutically acceptable carrier. In one embodiment the pharmaceutical preparation also contains leptin.

In a preferred embodiment the leptin is derived from a recombinant source. This aspect of the invention also provides pharmaceutical preparations containing a pharmaceutically acceptable carrier and an agonist of apolipoprotein J and/or an agonist to the apolipoprotein J-leptin binding complex.

This aspect of the invention also includes compositions comprising leptin bound to the leptin binding protein and compositions consisting essentially of leptin bound to the leptin binding protein. In preferred embodiments of the compositions and the pharmaceutical preparations that contain both leptin and the leptin binding protein, at least some of the leptin binding protein is bound to at least some of the leptin. In

more preferred embodiments, at least 75% of the leptin is bound to the leptin binding protein.

In its broadest range, for injection or infusion, dosage will be between 0.01 μg of biologically active protein/kg body weight, (calculating the mass of the protein alone, without chemical modification), and 10 mg/kg (based on the same). The dose of the composition which includes leptin is preferably between about 0.1 and 10 mg leptin/kg body weight. The dosing schedule may vary, depending on the circulation half-life of the protein or derivative used, whether the polypeptide is delivered by bolus dose or continuous infusion, and the formulation used.

The present invention also envisions methods of treating abnormalities in the endogenous leptin pathway in a mammalian subject by using multiple doses of the compositions and pharmaceutical preparations described herein. The "at least one dose" can be at least one dose per day for at least about five days. In a preferred embodiment, the at least one dose is at least one dose per day for at least about 30 days. The dose or doses can be administered by a parenteral route, such as an intravenous or intraperitoneal route. The method can further comprise the step of serially monitoring the amount of leptin bound to the leptin binding protein in test samples taken from the mammalian subject after the step of "administering". In preferred embodiments, this method also further comprises one or more determinations prior to the "administering" step.

In addition, the use of more than one composition and/or pharmaceutical preparation in a given treatment is also envisioned by the present invention. A specific embodiment of this aspect of the invention describes a method of treating an abnormality in the endogenous leptin pathway in a mammalian subject that comprises the steps of (a) administering to the mammalian subject a therapeutically effective amount of the composition comprising purified apolipoprotein J and a pharmaceutically acceptable carrier; and (b) administering to the mammalian subject a

therapeutically effective amount of leptin. As in all other aspects of the invention apolipoprotein J can serve as the leptin binding protein in the methods of diagnosing and treating described herein.

The present invention also includes methods for monitoring the progress of all of the treatments described herein. In one embodiment a method of monitoring treatment of an abnormality in the endogenous leptin pathway in a subject mammal comprising the step of administering at least one dose of a composition comprising leptin bound to apolipoprotein J, further comprises the step of serially monitoring a value determined for a property of a biological sample acquired from the mammalian subject, the property being selected from the group consisting of (i) the quantity of leptin bound to the leptin binding protein; (ii) the quantity of leptin not bound to the leptin binding protein; (iii) a quantitative relationship between (i) and (ii); and (iv) a quantitative relationship between the quantity of total leptin and alternative (i) or alternative (ii).

A related method of serially monitoring the progress of all of the treatments described herein monitors at least one of the following levels in a test sample, at least one time after the administering step: (i) an absolute amount of leptin bound to the leptin binding protein; (ii) an absolute amount of leptin not bound to the leptin binding protein; (iii) a percent of total leptin that is bound to the leptin binding protein; (iv) a percent of total leptin that is not bound to the leptin binding protein; (v) the ratio of (an absolute amount of leptin that is bound to the leptin binding protein) to (an absolute amount of total leptin); (vi) the ratio of (an absolute amount of leptin that is bound to the leptin binding protein) to (an absolute amount of leptin that is not bound to the leptin binding protein); (vii) the ratio of (an absolute amount of leptin that is not bound to the leptin binding protein) to (an absolute amount of total leptin); and (viii) a combination of the preceding.

The present invention also includes kits that aid in the practice of the present invention. In all of the kits of the present invention the leptin binding protein can be

apolipoprotein J. The present invention provides kits useful for performing all of the methods of detecting, quantitating and diagnosing described herein. One embodiment is a kit for detecting the presence of leptin that is associated with the leptin binding protein in a biological sample. The kit comprises a container holding a binding partner that has an affinity for leptin bound to the leptin binding protein. In a preferred embodiment, a container holding purified leptin bound to the leptin binding protein, for use as a standard, is also enclosed.

Another embodiment is a kit for performing the method of detecting leptin bound to apolipoprotein J in a biological sample comprising the steps of: (a) contacting an attached binding partner that has an affinity for leptin bound to apolipoprotein J with a biological sample, under conditions that allow the attached binding partner to associate with leptin bound to apolipoprotein J, and (b) contacting a detectable binding partner that has an affinity for the binding partner-leptin bound apolipoprotein J complex with the attached binding partner, and (c) separating unassociated detectable binding partner from the attached binding partner; and (d) detecting the detectable binding partner associated with the complex, wherein detecting the associated detectable binding partner can be correlated to detecting leptin bound to apolipoprotein J in a biological sample. Such a kit comprises (i) a container holding the attached-binding partner; and (ii) a container holding the detectable binding partner. In an alternative kit, the attached binding partner is already attached to a solid support. A container holding purified leptin for use as a standard, may also be enclosed in addition to, or in place of the container holding purified leptin. A container holding purified leptin in an admixture with the leptin binding protein, for use as a standard, may also be provided either with or without containers holding the purified leptin and/or the purified leptin binding protein standards. Enclosed protocols and/or buffers are also envisioned by the present invention as are components of some of the kit embodiments. These kits may also be used for monitoring the treatments of the present invention and for diagnosing an abnormality

in the endogenous leptin pathway in a mammalian subject by methods described herein.

These and other aspects of the present invention will be better appreciated by reference to the following drawings and Detailed Description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a plot obtained from running a sample containing human serum leptin, which had been previously eluted from anti-leptin affinity columns, on a gel filtration column performed at neutral pH. The continuous trace depicts the change in time in minutes (X-axis) *versus* the change in absorbance at 280 nm (Y-axis). The gel filtration column consisted of SUPEROSE 6 and SUPEROSE 12 packed into glass FPLC columns together in series, and pumped at 1 ml/min using standard HPLC equipment.

Figure 2 is a semi-continuous plot of immunoreactivity *versus* elution volume (fraction number) of fractions of human serum collected from the eluate of the gel filtration columns under non-dissociating conditions (neutral pH). The level of immunoreactive leptin in each fraction was measured by direct binding ELISA probed with biotinylated affinity purified polyclonal rabbit anti-human leptin antibodies after the addition of HRP-conjugated strepavidin, washing and the addition of the HRP substrate. The immunoreactivity is represented by the mean optical density reading attained after a ten minute incubation of the bound HRP and its substrate.

Figure 3 is a semi-continuous plot of immunoreactivity (ELISA) *versus* elution volume (fraction number) of fractions of human serum collected from the eluate of the gel filtration columns under dissociating conditions (0.1 M HCl containing 0.1 M NaCl). The level of immunoreactive leptin in each fraction was measured using a

polyclonal rabbit, anti-human leptin antibody-based immunoassay (closed squares (■)) and a mono-specific, polyclonal antibody-based assay (closed diamonds (♦)). The mono-specific, polyclonal antibody was raised to a synthetic peptide having an amino acid sequence common to mouse and human leptin but not found in known mouse or human proteins. The mean optical density was determined as described in Figure 2.

Figure 4 is a semi-continuous plot of immunoreactivity (ELISA) *versus* elution volume (fraction number) of fractions of antibody affinity purified mouse serum and human serum collected from the eluate of the gel filtration columns under dissociating conditions (0.1 M HCl containing 0.1 M NaCl). Immunoreactive low molecular weight (unbound) mouse serum leptin eluted from the column in the same numbered fractions (36-41) (closed diamonds (♦)) as did immunoreactive human serum leptin (open squares (□)). The mono-specific polyclonal antibody as described above was used as the probe. The mean optical density was determined as described in Figure 2.

Figure 5 is an SDS-PAGE analysis of the material recovered from a recombinant human leptin affinity column performed under non-reducing, Figure 5A (lanes 1 and 2) and reducing, Figure 5B (lanes 3 and 4) conditions (*i.e.*, in the presence of 2-mercaptoethanol), showing the band of leptin binding protein in the region of 40,000 daltons. Lanes 2 and 4 are molecular weight markers.

Figure 6 is a 5-15% SDS-PAGE analysis of whole, unfractionated normal human serum from nine different normal, non-obese individuals under non-reducing conditions Figure 6A and reducing conditions, Figure 6B. Lanes 1-9: normal samples; lane 10: molecular weight markers.

Figure 7 is a nitrocellulose immunoblot of the SDS-PAGE gel shown in Figure 6, using antiserum generated against unfractionated and unprocessed leptin binding

substance under both non-reducing conditions, Figure 7A and reducing conditions, Figure 7B.

Figure 8 is a nitrocellulose immunoblot of the SDS-PAGE gel shown in Figure 6, using affinity purified and cross-absorbed rabbit antiserum against human leptin binding substance under non-reducing conditions, Figure 8A and reducing conditions, Figure 8B.

Figure 9 is a 5-15% SDS-PAGE analysis under reducing conditions of four individual lanes which correspond to the four fractions collected sequentially from a recombinant human leptin affinity gel-filtration column. Lane 1 (leftmost) corresponded to the fraction with the highest molecular weight, lane 4 (from the left) corresponded to the fraction with the lowest molecular weight and lanes 2 and 3 corresponded to the fractions of intermediate weight. Lanes 1 and 2 show a predominant band in the molecular weight region of 40 kD, the weight corresponding to the leptin binding substance, and lanes 3 and 4 show predominantly low molecular weight contaminating materials.

Figure 10 is a scatter diagram summary of the results of 2 parallel immunoassays in 24 subjects for unassociated leptin (free L) and leptin associated with the leptin binding protein (L-LBP). The data are presented in terms of optical density (OD) units. Coordinates are given for An, anorexic (□), No, normals (●), Ob, obese (▵) and PW, Prader-Willi (*) subjects. The optical density measurements shown along the X axis represent a measure of the amount of leptin not associated with the leptin binding protein. The optical density measurements shown along the Y axis represent a measure of the amount of leptin associated with the leptin binding protein. Figure 10A was performed with human serum samples tested at a 1:20 dilution (MM 1:20). The upper left distribution corresponds to data from normal and lean anorexic patients. The lower right distribution corresponded to data from obese and Prader-Willi patients. Figure 10B was performed with human serum samples at a 1:2

dilution (MM 1:2). The far left distribution corresponds to data from normal and lean anorexic patients. The middle to right distribution corresponds to data from obese and Prader-Willi patients. The mean optical density was determined as described in Figure 2.

Figure 11A depicts a plot of the percentage of body fat determined from serum samples of individual controls (x-axis) *versus* the measured value for L/L-LBP, the ratio of leptin to leptin bound to the leptin binding protein (y-axis). The relative amounts of total leptin and the leptin bound to the leptin binding protein were determined as described for Example 2. Figure 11B depicts the identical relationships as Figure 11A except the serum samples were obtain from AIDS patients.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based in part on the discovery that a measurable fraction of blood- borne leptin in normal mammals is associated with a leptin binding protein. By virtue of the association between the leptin binding protein and a lipoprotein particle such as HDL, LDL, or VLDL, at least part of blood-borne leptin in mammals is bound to a lipoprotein complex. Apolipoprotein J, or a subclass of apolipoprotein J, is such a leptin binding protein.

One aspect of the present invention includes methods and compositions useful in detecting physiological obesity and other conditions related to abnormalities of the endogenous leptin pathway. Another aspect of the present invention includes methods and compositions useful in treating physiological obesity, and other conditions related to abnormalities of the endogenous leptin pathway. The present invention also includes methods of making these compositions and related pharmaceutical preparations.

The pharmaceutical preparations of the present invention may be prepared with a suitable carrier and at a strength effective for administration by various means to a patient experiencing abnormal fluctuations in body weight or adiposity. A variety of administrative techniques may be utilized, among them oral administration, nasal and other forms of transmucosal administration, parenteral techniques such as subcutaneous, intravenous and intraperitoneal injections, catheterizations and the like. Dosages of leptin binding protein, either alone or in an admixture with leptin, may vary and in particular should be based upon the recommendations and prescription of a qualified physician or veterinarian.

The discovery of the role of the leptin binding protein of the present invention has important implications for the diagnosis and treatment of nutritional disorders including, but not limited to, obesity, weight loss associated with cancer and AIDS and the treatment of diseases associated with obesity such as hypertension, heart disease, and Type II diabetes. In addition, there are potential agricultural uses for the leptin binding protein in cases where one might wish to modulate the body weight of domestic animals.

As used herein, "physiological obesity" and "physiologically obese" refer to excessive adipose tissue that is due at least in part to abnormalities in the endogenous leptin pathway, including abnormalities in leptin quantity, form (such as proportion that is bound) or effect. Abnormalities in the "endogenous leptin pathway" include deleterious deviations in the concentration of total leptin, or of a form of leptin, such as the concentration of leptin that is bound to a particular leptin binding protein. In addition, these abnormalities can include deleterious deviations in the function of leptin, such as a defective leptin protein or a defective leptin receptor. Abnormalities in the endogenous leptin pathway may be manifested in a number of ways including an abnormal food intake, an abnormal activity level, or an abnormal body temperature.

As used herein, "leptin" encompasses biologically active variants of naturally occurring leptin, as well as biologically active fragments of naturally occurring leptin and variants thereof, and combinations of the preceding. Leptin is the polypeptide product of the *ob* gene as described in the International Patent Publication No. WO 96/05309, and the US Patent Application No. 08/483,211 to which it claims priority, each of which is incorporated herein by reference in its entirety. Putative analogs and fragments of leptin are reported in US Patent 5,521,283, US Patent 5,532,336 and International Patent Publication No. PCT 96/22308 for International Application No. PCT/US96/ 01471, each of which is incorporated herein by reference in its entirety. Where leptin is used in the present invention, *e.g.*, in an admixture or complex with the leptin binding protein, all of the variants thereof, including fragments, derivatives, and analogs as defined in the aforementioned patent documents, are intended. Similarly, leptin and/or the leptin binding protein modified by conjugation to a carrier or polymer, or provided in a controlled release matrix, liposome, or other delivery vehicle, is intended according to the present invention.

Apolipoprotein J differs from other apolipoproteins by its molecular weight, subunit structure and isoelectric point [de Silva *et al.*, *J. Biol. Chem.* **265**: 14292-14297 (1990)]. As used herein, "apolipoprotein J" encompasses biologically active variants of naturally occurring apolipoprotein J, including monomeric, dimeric and oligomeric forms, deglycosylated forms as well as biologically active fragments of naturally occurring apolipoprotein J, subclasses thereof, variants thereof, and combinations of the preceding. The variants can include biologically active homodimeric forms of either the α or β subunits, and forms with various glycosylation patterns. Apolipoprotein J is an apolipoprotein made up of two non-identical monomers linked together by a disulfide bond. Both the dimeric protein and the individual subunits are purified to homogeneity from human plasma by standard protein purification procedures using immunoaffinity chromatography followed by reverse-phase HPLC. Whether the individual subunits or the dimer are isolated depends on whether the affinity chromatography eluate is reduced or not prior to the reverse-phase HPLC

fractionation [de Silva *et al.*, *J. Biol. Chem.* **265**: 14292-14297 (1990)]. The apparent molecular weight of the monomeric form of apolipoprotein J is about 40,000 daltons. This value is consistent with published values for the human ApoJ α subunit (34-36 kD) and the human ApoJ β subunit (36-39 kD). When chemically deglycosylated the reported molecular weights are 24 kD and 28 kD respectively [de Silva *et al.*, *Biochemistry*, **29**: 5380-5389 (1990)]. Both subunits of Apolipoprotein J are encoded by a single gene in the human and mouse genomes and is synthesized as a 427 amino acid polypeptide that is cleaved between Arg-205 and Ser-206 into two polypeptides: ApoJ α comprising amino acids 1-205 and ApoJ β comprising amino acids 205-427. The variants of the present invention, and as described above also include biologically active forms of the pro-apolipoprotein J (*i.e.*, the uncleaved parent polypeptide) as well as biologically active alternatively cleaved polypeptides, fragments thereof and combinations thereof. In addition, biologically active polypeptides containing additional N-terminal amino acids such as a single methionine, or several additional amino acids or even a fusion protein are also included in this definition as variants. The biologically active fragments of apolipoprotein J include the three amphiphilic helices identified for apolipoprotein J, namely Met-150 to Glu-167 of Apo Jα, and Met-221 to Asp-237 and Val-401 to Gln-418 of Apo Jβ, along with the potential Heparin-Binding domains as described by de Silva *et al.*, *Biochemistry* **29**, 5380-5389 (1990) which is hereby incorporated by reference in its entirety, and an additional amphiphilic helix identified in Rosenberg *et al.*, Int. J. of Cell Biol. **27**, 633-645 (1995) which is hereby incorporated by reference in its entirety.

"Subclasses" of apolipoprotein J, as used herein, are meant to distinguish apolipoprotein J that is isolated from one subclass of high density lipoprotein (HDL) from another. These subclasses of HDLs vary in size, charge, and composition, as well as in function.

The term "leptin binding protein" is used herein interchangeably with the term "leptin binding substance".

As used herein the terms "bound" or "binds" or "associates" or "associated" are meant to include all such specific interactions that result in two or more molecules showing a preference for one another relative to some third molecule. This includes processes such as covalent, ionic, hydrophobic and hydrogen bonding but does not include non-specific associations such solvent preferences. Such "bound" and "associated" leptin and leptin binding protein molecules may be referred to herein as a "complex."

As used herein, the phrase "conditions related to abnormalities of the endogenous leptin pathway" encompasses conditions and diseases due, at least in part, to abnormalities involving leptin as detailed above.

The term "medically assisting" is used herein as a manner of attending to the health care needs of a subject who has a particular problem (*e.g.*, an abnormality in the endogenous leptin pathway) which encompasses either diagnosing or treating that problem, and all combinations thereof. In one embodiment, the invention provides for medically assisting a mammalian subject suffering from an abnormality in the endogenous leptin pathway resulting in decreased leptin activity. In another embodiment, a mammalian subject may be suffering from an abnormality resulting in increased leptin activity. In each case, the decreased or increased leptin activity may be manifested as a pathological state, such as obesity (decreased leptin activity) or anorexia (increased leptin activity).

The term "detectable" is used broadly herein to include a factor that directly emits the detectable signal (such as a fluorescent molecule) an entity bound directly to that factor (such as an antibody carrying a fluorescent molecule) as well as a detectable target of the factor or the entity (such as an epitope of an antigen that reacts with the antibody carrying a fluorescent molecule). Thus a target may be termed detectable due to either its potential or realized association with either a factor that functions as a detectable label or a factor that is bound to a detectable label. Detectable labels, include but not limited to an enzyme, a radioactive element, a biochemiluminescent, a

chromophore that absorbs in the ultraviolet and/or visible and/or infrared region of the electromagnetic spectrum; and a fluorophore.

As defined herein, a "quantitative relationship" between two or more determinations of one or more substances, includes the relative absolute amounts, a relative percentage, a relative ratio, the difference, sum, multiple and/or quotient for two or more determinations; and can further include appropriate first or higher order equations that express the relationship between two or more determinations in a manner that can be understood by a person skilled in the art to which the present invention pertains.

A "determination" or "determining" as used herein is the result of an assay that includes an attempt to detect and/or the quantifying of the substance detected such as that expressed in a quantity or an amount of that substance.

As used herein the term "acquired from the mammalian subject" is meant to indicate the original source of the biological sample, that is the individual mammalian subject that the biological sample originates from.

As used herein the term "mono-specific antibody" describes an antibody to a protein raised against a particular peptide sequence in that protein and is meant to imply that the antibody is specific for an epitope that includes that particular sequence. A monospecific antibody may be either polyclonal or monoclonal.

Leptin Binding Protein

As used herein, apolipoprotein J (APO-J or NA1/NA2) has a number of other names that have arisen because of its apparent numerous physiological functions which include (1) clusterin, a protein from testis fluid with cell aggregating activity, (2) sulfated glycoprotein 2, a major sulfate Sertoli cell secretion product, (3) the cytolysis inhibitor or complement lysis inhibitor, SP40 or CLI, a constituent of the soluble

nonlytic terminal complement complex (non-cell-associated), (4) T64, an mRNA found induced in neuroretinal cells by Rous Sarcoma Virus, (5) testosterone-repressed prostatic messenger 2, characterized as mRNA induced during involution of the prostate after androgen withdrawal (6) gp-III, a constituent of chromaffin granules, (7) gp-80, a glycoprotein secreted at the apical surface of kidney epithelial cells, (8) pADHC-9, an mRNA from the hippocampus of a patient suffering from Alzheimer's disease, (9) pTB16, a cDNA clone isolated from glioma library and an mRNA highly expressed in epileptic foci, and HISL-19 or Secretogranin IV, an immunocytochemical marker of neuroendocrine cells. [Jenne & Tschopp, *Trends in Biochemical Sciences*, **196**, vol. 17, no. 4 (1992)].

The cDNA and deduced amino acid sequences of apolipoprotein J have been published by de Silva *et al.*, *Biochemistry* **29**:5380-5389 (1990), which is hereby incorporated by reference. The mouse apolipoprotein J has been published by Jordan-Starck *et al.*, *Journal of Lipid Research* **35**:194-210 (1994), hereby incorporated by reference. Apolipoprotein J has been purified by a number of different techniques. One such method is described in *Methods of Enzymology* **263**:309-316, which is hereby incorporated by reference [Jenkins *et al.*, *supra*].

Isolation of Leptin Binding Protein/Leptin-LBP Complex

A solid support for use in the present invention will be inert to the reaction conditions for binding. A solid support for use in the present invention may have reactive groups in order to attach a binding partner, such as an antibody to the leptin binding protein. In another embodiment, the solid support may be a useful chromatographic support, such as the carbohydrate polymers SEPHAROSE, SEPHADEX, and agarose. As used herein, a solid support is not limited to a specific type of support. Rather, a large number of supports are available and are known to one of ordinary skill in the art. Solid supports include silica gels, resins, derivatized plastic films, glass beads, cotton, plastic beads, alumina gels, magnetic beads, membranes (including but not limited to nitrocellulose, cellulose, nylon, and glass wool filters), plastic and glass dishes or

wells, etc. For example, solid supports used for peptide or oligonucleotide synthesis can be used, such as polystyrene resin (*e.g.*, PAM-resin obtained from Bachem Inc., Peninsula Laboratories, etc.), POLYHIPE® resin (obtained from Aminotech, Canada), polyamide resin (obtained from Peninsula Laboratories), polystyrene resin grafted with polyethylene glycol (TentaGel®, Rapp Polymere, Tubingen, Germany) or polydimethylacrylamide resin (obtained from Milligen/Biosearch, California). The solid support can be formulated as a chromatography support, *e.g.*, in a column; it can be used in suspension followed by filtration, sedimentation, magnetic association, or centrifugation; or by washing, as in a membrane, well, plastic film, etc.

Analogs of the leptin binding protein

The present invention specifically contemplates preparation of analogs of the leptin binding protein that function similarly to the leptin binding protein. Preferably, a leptin binding protein agonist is more effective than the native protein. For example, a leptin binding protein agonist analog may demonstrate a longer half-life *in vivo*. Nevertheless, leptin binding protein agonist analogs that are less effective than the native protein are also contemplated. In another embodiment, the analog antagonizes leptin activity. For example, the leptin binding protein analog that binds to leptin but prevents leptin from binding to the leptin receptor can potentially decrease leptin activity *in vivo*.

One such embodiment of this aspect of the invention is an analog of the leptin binding protein that is the apolipoprotein J modified by substitution of amino acids at positions on the polypeptide that are not essential for its function as a leptin binding protein. In the long term, the elucidation of the biochemical function of the leptin binding protein is useful for identifying small molecule agonists and antagonists that affect its activity and its effect on leptin.

Derivatives of the leptin binding protein

Generally, the leptin binding protein, especially apolipoprotein J, may be derivatized by the attachment of one or more chemical moieties to the protein moiety, either alone or in a complex with leptin. The chemically modified derivatives may be further formulated for intraarterial, intraperitoneal, intramuscular, subcutaneous, intravenous, oral, nasal, rectal, buccal, sublingual, pulmonary, topical, trans-dermal, or other routes of administration. Chemical modification of biologically active proteins has been found to provide additional advantages under certain circumstances, such as increasing the stability and circulation time of the therapeutic protein and decreasing immunogenicity. *See* U.S. Patent No. 4,179,337, Davis *et al.*, issued December 18, 1979. For a review, *see* Abuchowski *et al.*, "Soluble Polymer-Enzyme Adducts", in *Enzymes as Drugs*, pp. 367-383, Holcenberg and Roberts, eds., Wiley-Interscience, New York, NY, (1981). A review article describing protein modification and fusion proteins is Francis, *Focus on Growth Factors*, 3:4-10 (1992).

Chemical Moieties For Derivatization. The chemical moieties suitable for derivatization may be selected from among water soluble polymers. The polymer selected should be water soluble so that the protein to which it is attached does not precipitate in an aqueous environment, such as a physiological environment. Preferably, for therapeutic use of the end-product preparation, the polymer will be pharmaceutically acceptable. One skilled in the art will be able to select the desired polymer based on such considerations as whether the polymer/protein conjugate will be used therapeutically, and if so, the desired dosage, circulation time, resistance to proteolysis, and other considerations. For the leptin bind protein and the (L-LBP) complex, these may be ascertained using the assays provided herein.

Polymer Molecules. The water soluble polymer may be selected from the group consisting of, for example, polyethylene glycol, copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic

anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propylene glycol homopolymers, polypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols and polyvinyl alcohol. Polyethylene glycol propionaldenhyde may provide advantages in manufacturing due to its stability in water.

The polymer may be of any molecular weight, and may be branched or unbranched. For polyethylene glycol, the preferred molecular weight is between about 2 kD and about 100 kD (the term "about" indicating that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight) for ease in handling and manufacturing. Other sizes may be used, depending on the desired therapeutic profile (*e.g.*, the duration of sustained release desired, the effects, if any on biological activity, the ease in handling, the degree or lack of antigenicity and other known effects of the polyethylene glycol to a therapeutic protein or analog).

Polymer/Protein Ratio. The number of polymer molecules so attached may vary, and one skilled in the art will be able to ascertain the effect on function. One may mono-derivatize, or may provide for a di-, tri-, tetra- or some combination of derivatization, with the same or different chemical moieties (*e.g.*, polymers, such as different weights of polyethylene glycols). The proportion of polymer molecules to protein (or peptide) molecules will vary, as will their concentrations in the reaction mixture. In general, the optimum ratio (in terms of efficiency of reaction in that there is no excess un-reacted protein or polymer) will be determined by factors such as the desired degree of derivatization (e.g., mono, di-, tri-, etc.), the molecular weight of the polymer selected, whether the polymer is branched or unbranched, and the reaction conditions.

Attachment of the Chemical Moiety to the Protein. The polyethylene glycol molecules (or other chemical moieties) should be attached to the protein with consideration of effects on functional or antigenic domains of the protein. There are a

number of attachment methods available to those skilled in the art, *e.g.*, EP 0 401 384 herein incorporated by reference (coupling PEG to G-CSF). *See also* Malik *et al.*, *Exp. Hematol.*, **20**:1028-1035 (1992) (reporting pegylation of GM-CSF using tresyl chloride). For example, polyethylene glycol may be covalently bound through amino acid residues via a reactive group, such as a free amino or carboxyl group. Reactive groups are those to which an activated polyethylene glycol molecule may be bound. The amino acid residues having a free amino group may include lysine residues and the N-terminal amino acid residues, those having a free carboxyl group may include aspartic acid residues glutamic acid residues and the C-terminal amino acid residue. Sulfhydryl groups may also be used as a reactive group for attaching the polyethylene glycol molecule(s). Preferred for therapeutic purposes is attachment at an amino group, such as attachment at the N-terminus or lysine group. Attachment at residues important for receptor binding should be avoided if receptor binding is desired.

N-terminally Chemically Modified Proteins. One may specifically desire N-terminally chemically modified protein. Using polyethylene glycol as an illustration of the present compositions, one may select from a variety of polyethylene glycol molecules (by molecular weight, branching, etc.), the proportion of polyethylene glycol molecules to protein (or peptide) molecules in the reaction mix, the type of pegylation reaction to be performed, and the method of obtaining the selected N-terminally pegylated protein. The method of obtaining the N-terminally pegylated preparation (*i.e.*, separating this moiety from other monopegylated moieties if necessary) may be by purification of the N-terminally pegylated material from a population of pegylated protein molecules. Selective N-terminal chemical modification may be accomplished by reductive alkylation, which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminus) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of the protein at the N-terminus with a carbonyl group containing polymer is achieved. For example, one may selectively N-terminally pegylate the protein by performing the reaction at a pH which allows

one to take advantage of the pK_a differences between the ϵ-amino groups of the lysine residues and that of the α-amino group of the N-terminal residue of the protein. By such selective derivatization attachment of a water soluble polymer to a protein is controlled: the conjugation with the polymer takes place predominantly at the N-terminus of the protein and no significant modification of other reactive groups, such as the lysine side chain amino groups, occurs. Using reductive alkylation, the water soluble polymer may be of the type described above, and should have a single reactive aldehyde for coupling to the protein. Polyethylene glycol propionaldehyde, containing a single reactive aldehyde, may be used.

Expression of Leptin Binding Protein/Leptin-LBP Complex

The functional activity of the leptin binding protein alone or with leptin can be evaluated transgenically. In this respect, a transgenic mouse model can be used. Transgenic vectors, including viral vectors, or cosmid clones (or phage clones) corresponding to the wild type locus of a candidate gene, can be constructed using the isolated apolipoprotein J gene, for example. Cosmids may be introduced into transgenic mice using published procedures [Jaenisch, *Science*, **240**:1468-1474 (1988)].

The apolipoprotein J gene can be tested by examining its phenotypic effects when expressed in antisense orientation in wild-type animals. In this approach, expression of the wild-type allele is suppressed, which leads to a mutant phenotype. RNA·RNA duplex formation (antisense-sense) prevents normal handling of mRNA, resulting in partial or complete elimination of wild-type gene effect. This technique has been used to inhibit TK synthesis in tissue culture and to produce phenotypes of the *Kruppel* mutation in *Drosophila*, and the *Shiverer* mutation in mice [Izant *et al.*, *Cell*, **36**:1007-1015 (1984); Green *et al.*, *Annu. Rev. Biochem.*, **55**:569-597 (1986); Katsuki *et al.*, *Science*, **241**:593-595 (1988)]. An important advantage of this approach is that only a small portion of the gene need be expressed for effective inhibition of expression of the entire cognate mRNA. The antisense transgene will be placed under

control of its own promoter or another promoter expressed in the correct cell type, and placed upstream of the SV40 polyA site. This transgene will be used to make transgenic mice. This procedure can also be repeated in mice having a non-functional *OB* gene.

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Genes Encoding Leptin Binding Proteins

The present invention contemplates isolation of a gene encoding a leptin binding protein of the invention, including a full length, or naturally occurring form of a leptin binding protein, and any antigenic fragments thereof from any animal, particularly 10 mammalian or avian, and more particularly human, source for use in the invention as defined herein. As used herein, the term "gene" refers to an assembly of nucleotides that encode a polypeptide, and includes cDNA and genomic DNA nucleic acids. One example of a leptin binding protein of the present invention is apolipoprotein J. The human sequence (SEQ ID NO:2) is disclosed in de Silva *et al.* (1990) *Biochemistry* 15 **29**, 5380-5389 and is incorporated by reference in its entirety herein. The mouse sequence (SEQ ID NO:3 is disclosed in Jordan-Starck *et al.* (1994) J. of Lipid Research **35** 194-210 and is incorporated by reference in its entirety herein.

In accordance with the present invention there may be employed conventional 20 molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook et al., 1989"); *DNA Cloning: A Practical Approach*, Volumes I and II 25 (D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Nucleic Acid Hybridization* [B.D. Hames & S.J. Higgins eds. (1985)]; *Transcription And Translation* [B.D. Hames & S.J. Higgins, eds. (1984)]; *Animal Cell Culture* [R.I. Freshney, ed. (1986)]; *Immobilized Cells And Enzymes* [IRL Press, (1986)]; B. Perbal, *A Practical Guide To Molecular Cloning* (1984); F.M. Ausubel et al. (eds.), *Current* 30 *Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994).

Therefore, if appearing herein, the following terms shall have the definitions set out below.

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment. A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*, *i.e.*, capable of replication under its own control.

A "cassette" refers to a segment of DNA that can be inserted into a vector at specific restriction sites. The segment of DNA encodes a polypeptide of interest, and the cassette and restriction sites are designed to ensure insertion of the cassette in the proper reading frame for transcription and translation.

A cell has been "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. A cell has been "transformed" by exogenous or heterologous DNA when the transfected DNA effects a phenotypic change. Preferably, the transforming DNA should be integrated (covalently linked) into chromosomal DNA making up the genome of the cell.

"Heterologous" DNA refers to DNA not naturally located in the cell, or in a chromosomal site of the cell. Preferably, the heterologous DNA includes a gene foreign to the cell.

A "nucleic acid molecule" refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules"), or any phosphoester analogs thereof, such as phosphorothioates and thioesters, in either single stranded form, or a double-stranded helix. Double stranded DNA-DNA, DNA-RNA and RNA-RNA helices are possible.

The term nucleic acid molecule, and in particular DNA or RNA molecule, refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear or circular DNA molecules (*e.g.*, restriction fragments), plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5′ to 3′ direction along the nontranscribed strand of DNA (*i.e.*, the strand having a sequence homologous to the mRNA). A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation.

A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength (*see* Sambrook et al., *supra*). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. For preliminary screening for homologous nucleic acids, low stringency hybridization conditions, corresponding to a T_m of 55°, can be used, *e.g.*, 5x SSC, 0.1% SDS, 0.25% milk, and no formamide; or 30% formamide, 5x SSC, 0.5% SDS). Moderate stringency hybridization conditions correspond to a higher T_m, *e.g.*, 40% formamide, with 5x or 6x SCC. High stringency hybridization conditions correspond to the highest T_m, *e.g.*, 50% formamide, 5x or 6x SCC. Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the value of T_m for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher T_m) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA.

For hybrids of greater than 100 nucleotides in length, equations for calculating T_m have been derived (*see* Sambrook et al., *supra*, 9.50-0.51). For hybridization with shorter nucleic acids, *i.e.*, oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (*see* Sambrook et al., *supra*, 11.7-11.8). Preferably a minimum length for a hybridizable nucleic acid is at least about 10 nucleotides; preferably at least about 15 nucleotides; and more preferably the length is at least about 20 nucleotides.

In a specific embodiment, the term "standard hybridization conditions" refers to a T_m of 55°C, and utilizes conditions as set forth above. In a preferred embodiment, the T_m is 60°C; in a more preferred embodiment, the T_m is 65°C.

"Homologous recombination" refers to the insertion of a foreign DNA sequence of a vector in a chromosome. Preferably, the vector targets a specific chromosomal site for homologous recombination. For specific homologous recombination, the vector will contain sufficiently long regions of homology to sequences of the chromosome to allow complementary binding and incorporation of the vector into the chromosome. Longer regions of homology, and greater degrees of sequence similarity, may increase the efficiency of homologous recombination.

A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide in a cell *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5′ (amino) terminus and a translation stop codon at the 3′ (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (*e.g.*, mammalian) DNA, and even synthetic DNA sequences. If the coding sequence is intended for expression in a eukaryotic cell, a polyadenylation signal and transcription termination sequence will usually be located 3′ to the coding sequence.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, terminators, and the like, that provide for the expression of a coding sequence in a host cell. In eukaryotic cells, polyadenylation signals are control sequences.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3′ direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3′ terminus by the transcription initiation site and extends upstream (5′ direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then trans-RNA spliced and translated into the protein encoded by the coding sequence.

A "signal sequence" is included at the beginning of the coding sequence of a protein to be expressed on the surface of a cell. This sequence encodes a signal peptide, N-terminal to the mature polypeptide, that directs the host cell to translocate the polypeptide. The term "translocation signal sequence" is used herein to refer to this sort of signal sequence. Translocation signal sequences can be found associated with a variety of proteins native to eukaryotes and prokaryotes, and are often functional in both types of organisms.

As used herein, the term "sequence homology" in all its grammatical forms refers to the relationship between proteins that possess a "common evolutionary origin,"

including proteins from superfamilies (*e.g.*, the immunoglobulin superfamily) and homologous proteins from different species (*e.g.*, myosin light chain, etc.) (Reeck et al., 1987, Cell 50:667).

Accordingly, the term "sequence similarity" in all its grammatical forms refers to the degree of identity or correspondence between nucleic acid or amino acid sequences of proteins that do not share a common evolutionary origin (*see* Reeck et al., *supra*). However, in common usage and in the instant application, the term "homologous," when modified with an adverb such as "highly," may refer to sequence similarity and not a common evolutionary origin.

In a specific embodiment, two DNA sequences are "substantially homologous" or "substantially similar" when at least about 50% (preferably at least about 75%, and most preferably at least about 90 or 95%) of the nucleotides match over the defined length of the DNA sequences. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Maniatis et al., *supra*; DNA Cloning, Vols. I & II, *supra*; Nucleic Acid Hybridization, *supra*.

Similarly, in a particular embodiment, two amino acid sequences are "substantially homologous" or "substantially similar" when greater than 30% of the amino acids are identical, or greater than about 60% are similar (functionally identical). Preferably, the similar or homologous sequences are identified by alignment using, for example, the GCG (Genetics Computer Group, Program Manual for the GCG Package, *Version 7*, Madison, Wisconsin) pileup program.

The term "corresponding to" is used herein to refer similar or homologous sequences, whether the exact position is identical or different from the molecule to which the

similarity or homology is measured. The term "corresponding to" refers to the sequence similarity, and not the numbering of the amino acid residues or nucleotide bases.

A gene encoding a leptin binding protein, whether genomic DNA or cDNA, can be isolated from any source, particularly from a human cDNA or genomic library. Methods for obtaining a leptin binding protein gene are well known in the art, as described above (*see, e.g.*, Sambrook et al., 1989, *supra*).

Accordingly, any animal cell potentially can serve as the nucleic acid source for the molecular cloning of a *leptin binding protein* gene. The DNA may be obtained by standard procedures known in the art from cloned DNA (*e.g.*, a DNA "library"), and preferably is obtained from a cDNA library prepared from tissues with high level expression of the protein, by chemical synthesis, by cDNA cloning, or by the cloning of genomic DNA, or fragments thereof, purified from the desired cell (See, for example, Sambrook et al., 1989, *supra*; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II). Clones derived from genomic DNA may contain regulatory and intron DNA regions in addition to coding regions; clones derived from cDNA will not contain intron sequences. Whatever the source, the gene should be molecularly cloned into a suitable vector for propagation of the gene.

In the molecular cloning of the gene from genomic DNA, DNA fragments are generated, some of which will encode the desired gene. The DNA may be cleaved at specific sites using various restriction enzymes. Alternatively, one may use DNAse in the presence of manganese to fragment the DNA, or the DNA can be physically sheared, as for example, by sonication. The linear DNA fragments can then be separated according to size by standard techniques, including but not limited to, agarose and polyacrylamide gel electrophoresis and column chromatography.

Once the DNA fragments are generated, identification of the specific DNA fragment containing the desired *leptin binding protein* gene may be accomplished in a number of ways. For example, if an amount of a portion of a *leptin binding protein* gene or its specific RNA, or a fragment thereof, is available and can be purified and labeled, the generated DNA fragments may be screened by nucleic acid hybridization to the labeled probe (Benton and Davis, 1977, Science 196:180; Grunstein and Hogness, 1975, Proc. Natl. Acad. Sci. U.S.A. 72:3961). For example, a set of oligonucleotides corresponding to the partial amino acid sequence information obtained for the leptin binding protein can be prepared and used as probes for DNA encoding leptin binding protein, as primers for cDNA or mRNA (*e.g.*, in combination with a poly-T primer for RT-PCR). Preferably, a fragment is selected that is highly unique to leptin binding protein of the invention. Those DNA fragments with substantial homology to the probe will hybridize. As noted above, the greater the degree of homology, the more stringent hybridization conditions can be used. In a specific embodiment, stringency hybridization conditions are used to identify a homologous *leptin binding protein* gene.

Further selection can be carried out on the basis of the properties of the gene, *e.g.*, if the gene encodes a protein product having the isoelectric, electrophoretic, amino acid composition, or partial amino acid sequence of leptin binding protein protein as disclosed herein. Thus, the presence of the gene may be detected by assays based on the physical, chemical, or immunological properties of its expressed product. For example, cDNA clones, or DNA clones which hybrid-select the proper mRNAs, can be selected which produce a protein that, *e.g.*, has similar or identical electrophoretic migration, isoelectric focusing or non-equilibrium pH gel electrophoresis behavior, proteolytic digestion maps, or antigenic properties as known for leptin binding protein.

A *leptin binding protein* gene of the invention can also be identified by mRNA selection, *i.e.*, by nucleic acid hybridization followed by *in vitro* translation. In this

procedure, nucleotide fragments are used to isolate complementary mRNAs by hybridization. Such DNA fragments may represent available, purified *leptin binding protein* DNA, or may be synthetic oligonucleotides designed from the partial amino acid sequence information. Immunoprecipitation analysis of the *in vitro* translation products of the products of the isolated mRNAs identifies the mRNA and, therefore, the complementary DNA fragments, that contain the desired sequences. In addition, specific mRNAs may be selected by adsorption of polysomes isolated from cells to immobilized antibodies specifically directed against leptin binding protein.

A radiolabeled *leptin binding protein* cDNA can be synthesized using the selected mRNA (from the adsorbed polysomes) as a template. The radiolabeled mRNA or cDNA may then be used as a probe to identify homologous *leptin binding protein* DNA fragments from among other genomic DNA fragments.

The present invention also relates to cloning vectors containing genes encoding analogs and derivatives of leptin binding protein of the invention, that have the same or homologous functional activity as leptin binding protein, and homologs thereof from other species. The production and use of derivatives and analogs related to leptin binding protein are within the scope of the present invention.

Leptin binding protein derivatives can be made by altering encoding nucleic acid sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. Preferably, derivatives are made that have enhanced or increased functional activity relative to native leptin binding protein.

Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as a *leptin binding protein* gene may be used in the practice of the present invention. These include but are not limited to allelic genes, homologous genes from other species, and nucleotide sequences comprising all or portions of *leptin binding protein* genes which are altered by the

substitution of different codons that encode the same amino acid residue within the sequence, thus producing a silent change. Likewise, the leptin binding protein derivatives of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence of a leptin binding protein protein including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a conservative amino acid substitution. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity, which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. Amino acids containing aromatic ring structures are phenylalanine, tryptophan, and tyrosine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Such alterations will not be expected to affect apparent molecular weight as determined by polyacrylamide gel electrophoresis, or isoelectric point.

Particularly preferred substitutions are:

- Lys for Arg and vice versa such that a positive charge may be maintained;
- Glu for Asp and vice versa such that a negative charge may be maintained;
- Ser for Thr such that a free -OH can be maintained; and
- Gln for Asn such that a free NH_2 can be maintained.

Amino acid substitutions may also be introduced to substitute an amino acid with a particularly preferable property. For example, a Cys may be introduced a potential site for disulfide bridges with another Cys. A His may be introduced as a particularly "catalytic" site (i.e., His can act as an acid or base and is the most common amino acid

in biochemical catalysis). Pro may be introduced because of its particularly planar structure, which induces β-turns in the protein's structure.

The genes encoding leptin binding protein derivatives and analogs of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, the cloned leptin binding protein gene sequence can be modified by any of numerous strategies known in the art (Sambrook et al., 1989, *supra*). The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*. In the production of the gene encoding a derivative or analog of leptin binding protein, care should be taken to ensure that the modified gene remains within the same translational reading frame as the leptin binding protein gene, uninterrupted by translational stop signals, in the gene region where the desired activity is encoded.

Additionally, the leptin binding protein-encoding nucleic acid sequence can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Preferably, such mutations enhance the functional activity of the mutated leptin binding protein gene product. Any technique for mutagenesis known in the art can be used, including but not limited to, *in vitro* site-directed mutagenesis (Hutchinson, C., et al., 1978, J. Biol. Chem. 253:6551; Zoller and Smith, 1984, DNA 3:479-488; Oliphant et al., 1986, Gene 44:177; Hutchinson et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:710), use of TAB® linkers (Pharmacia), etc. PCR techniques are preferred for site directed mutagenesis (see Higuchi, 1989, "Using PCR to Engineer DNA", in *PCR Technology: Principles and Applications for DNA Amplification*, H. Erlich, ed., Stockton Press, Chapter 6, pp. 61-70).

The identified and isolated gene can then be inserted into an appropriate cloning vector. A large number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Examples of vectors include, but are not limited to, *E. coli*, bacteriophages such as lambda derivatives, or plasmids such as pBR322 derivatives or pUC plasmid derivatives, *e.g.*, pGEX vectors, pmal-c, pFLAG, etc. The insertion into a cloning vector can, for example, be accomplished by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc., so that many copies of the gene sequence are generated. Preferably, the cloned gene is contained on a shuttle vector plasmid, which provides for expansion in a cloning cell, *e.g.*, *E. coli*, and facile purification for subsequent insertion into an appropriate expression cell line, if such is desired. For example, a shuttle vector, which is a vector that can replicate in more than one type of organism, can be prepared for replication in both *E. coli* and *Saccharomyces cerevisiae* by linking sequences from an *E. coli* plasmid with sequences form the yeast 2μ plasmid.

In an alternative method, the desired gene may be identified and isolated after insertion into a suitable cloning vector in a "shot gun" approach. Enrichment for the desired gene, for example, by size fractionation, can be done before insertion into the cloning vector.

Expression of leptin binding protein Polypeptides

The nucleotide sequence coding for leptin binding protein, or antigenic fragment, derivative or analog thereof, or a functionally active derivative, including a chimeric protein, thereof, can be inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. Such elements are termed herein a "promoter." Thus, the nucleic acid encoding leptin binding protein of the invention is operationally associated with a promoter in an expression vector of the invention. Both cDNA and genomic sequences can be cloned and expressed under control of such regulatory sequences. An expression vector also preferably includes a replication origin.

The necessary transcriptional and translational signals can be provided on a recombinant expression vector, or they may be supplied by the native gene encoding leptin binding protein and/or its flanking regions.

Potential host-vector systems include but are not limited to mammalian cell systems infected with virus (*e.g.*, vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (*e.g.*, baculovirus); microorganisms such as yeast containing yeast vectors; or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used.

A recombinant leptin binding protein protein of the invention, or functional fragment, derivative, chimeric construct, or analog thereof, may be expressed chromosomally, after integration of the coding sequence by recombination. In this regard, any of a number of amplification systems may be used to achieve high levels of stable gene expression (*See* Sambrook et al., 1989, *supra*).

The cell into which the recombinant vector comprising the nucleic acid encoding leptin binding protein is cultured in an appropriate cell culture medium under conditions that provide for expression of leptin binding protein by the cell.

Any of the methods previously described for the insertion of DNA fragments into a cloning vector may be used to construct expression vectors containing a gene consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombination (genetic recombination).

Expression of leptin binding protein protein may be controlled by any promoter/enhancer element known in the art, but these regulatory elements must be functional in the host selected for expression. Promoters which may be used to control leptin binding protein gene expression include, but are not limited to, the SV40 early promoter region (Benoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42); prokaryotic expression vectors such as the β-lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the *tac* promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25); see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter; and the animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan,

1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58), alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94), myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712), myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

Expression vectors containing a nucleic acid encoding a leptin binding protein of the invention can be identified by four general approaches: (a) PCR amplification of the desired plasmid DNA or specific mRNA, (b) nucleic acid hybridization, (c) presence or absence of selection marker gene functions, and (d) expression of inserted sequences. In the first approach, the nucleic acids can be amplified by PCR to provide for detection of the amplified product. In the second approach, the presence of a foreign gene inserted in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to an inserted marker gene. In the third approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "selection marker" gene functions (*e.g.*, β-galactosidase activity, thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. In another

example, if the nucleic acid encoding leptin binding protein is inserted within the "selection marker" gene sequence of the vector, recombinants containing the leptin binding protein insert can be identified by the absence of the leptin binding protein gene function. In the fourth approach, recombinant expression vectors can be identified by assaying for the activity, biochemical, or immunological characteristics of the gene product expressed by the recombinant, provided that the expressed protein assumes a functionally active conformation.

A wide variety of host/expression vector combinations may be employed in expressing the DNA sequences of this invention. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences. Suitable vectors include derivatives of SV40 and known bacterial plasmids, e.g., *E. coli* plasmids col El, pCR1, pBR322, pMal-C2, pET, pGEX (Smith *et al.*, 1988, Gene 67:31-40), pMB9 and their derivatives, plasmids such as RP4; phage DNAS, e.g., the numerous derivatives of phage λ, e.g., NM989, and other phage DNA, e.g., M13 and filamentous single stranded phage DNA; yeast plasmids such as the 2μ plasmid or derivatives thereof; vectors useful in eukaryotic cells, such as vectors useful in insect or mammalian cells; vectors derived from combinations of plasmids and phage DNAs, such as plasmids that have been modified to employ phage DNA or other expression control sequences; and the like.

For example, in a baculovirus expression systems, both non-fusion transfer vectors, such as but not limited to pVL941 (*Bam*H1 cloning site; Summers), pVL1393 (*Bam*H1, *Sma*I, *Xba*I, *Eco*R1, *Not*I, *Xma*III, *Bgl*II, and *Pst*I cloning site; Invitrogen), pVL1392 (*Bgl*II, *Pst*I, *Not*I, *Xma*III, *Eco*RI, *Xba*I, *Sma*I, and *Bam*H1 cloning site; Summers and Invitrogen), and pBlue*Bac*III (*Bam*H1, *Bgl*II, *Pst*I, *Nco*I, and *Hind*III cloning site, with blue/white recombinant screening possible; Invitrogen), and fusion transfer vectors, such as but not limited to pAc700 (*Bam*H1 and *Kpn*I cloning site, in which the *Bam*H1 recognition site begins with the initiation codon; Summers), pAc701 and pAc702 (same as pAc700, with different reading frames), pAc360

(*Bam*H1 cloning site 36 base pairs downstream of a polyhedrin initiation codon; Invitrogen(195)), and pBlueBacHisA, B, C (three different reading frames, with *Bam*H1, *Bgl*II, *Pst*I, *Nco*I, and *Hind*III cloning site, an N-terminal peptide for ProBond purification, and blue/white recombinant screening of plaques; Invitrogen (220)) can be used.

Mammalian expression vectors contemplated for use in the invention include vectors with inducible promoters, such as the dihydrofolate reductase (DHFR) promoter, *e.g.*, any expression vector with a *DHFR* expression vector, or a *DHFR*/methotrexate co-amplification vector, such as pED (*Pst*I, *Sal*I, *Sba*I, *Sma*I, and *Eco*RI cloning site, with the vector expressing both the cloned gene and *DHFR*; *see* Kaufman, *Current Protocols in Molecular Biology*, 16.12 (1991). Alternatively, a glutamine synthetase/methionine sulfoximine co-amplification vector, such as pEE14 (*Hind*III, *Xba*I, *Sma*I, *Sba*I, *Eco*RI, and *Bcl*I cloning site, in which the vector expresses glutamine synthase and the cloned gene; Celltech). In another embodiment, a vector that directs episomal expression under control of Epstein Barr Virus (EBV) can be used, such as pREP4 (*Bam*H1, *Sfi*I, *Xho*I, *Not*I, *Nhe*I, *Hind*III, *Nhe*I, *Pvu*II, and *Kpn*I cloning site, constitutive RSV-LTR promoter, hygromycin selectable marker; Invitrogen), pCEP4 (*Bam*H1, *Sfi*I, *Xho*I, *Not*I, *Nhe*I, *Hind*III, *Nhe*I, *Pvu*II, and *Kpn*I cloning site, constitutive hCMV immediate early gene, hygromycin selectable marker; Invitrogen), pMEP4 (*Kpn*I, *Pvu*I, *Nhe*I, *Hind*III, *Not*I, *Xho*I, *Sfi*I, *Bam*H1 cloning site, inducible methallothionein IIa gene promoter, hygromycin selectable marker; Invitrogen), pREP8 (*Bam*H1, *Xho*I, *Not*I, *Hind*III, *Nhe*I, and *Kpn*I cloning site, RSV-LTR promoter, histidinol selectable marker; Invitrogen), pREP9 (*Kpn*I, *Nhe*I, *Hind*III, *Not*I, *Xho*I, *Sfi*I, and BamHI cloning site, RSV-LTR promoter, G418 selectable marker; Invitrogen), and pEBVHis (RSV-LTR promoter, hygromycin selectable marker, N-terminal peptide purifiable via ProBond resin and cleaved by enterokinase; Invitrogen). Selectable mammalian expression vectors for use in the invention include pRc/CMV (*Hind*III, *Bst*XI, *Not*I, *Sba*I, and *Apa*I cloning site, G418 selection; Invitrogen), pRc/RSV (*Hind*III, *Spe*I, *Bst*XI, *Not*I, *Xba*I cloning site, G418 selection;

Invitrogen), and others. Vaccinia virus mammalian expression vectors (*see*, Kaufman. 1991, *supra*) for use according to the invention include but are not limited to pSC11 (*Sma*I cloning site, TK- and β-gal selection), pMJ601 (*Sal*I, *Sma*I, *Afl*I, *Nar*I, *Bsp*MII, *Bam*HI, *Apa*I, *Nhe*I, *Sac*II, *Kpn*I, and *Hind*III cloning site; TK- and β-gal selection), and pTKgptF1S (*Eco*RI, *Pst*I, *Sal*I, *Acc*I, *Hind*II, *Sba*I, *Bam*HI, and Hpa cloning site, TK or XPRT selection).

Yeast expression systems can also be used according to the invention to express the leptin binding polypeptide. For example, the non-fusion pYES2 vector (*Xba*I, *Sph*I, *Sho*I, *Not*I, *Gst*XI, *Eco*RI, *Bst*XI, *Bam*H1, *Sac*I, *Kpn*1, and *Hind*III cloning sit; Invitrogen) or the fusion pYESHisA, B, C (*Xba*I, *Sph*I, *Sho*I, *Not*I, *Bst*XI, *Eco*RI, *Bam*H1, *Sac*I, *Kpn*I, and *Hind*III cloning site, N-terminal peptide purified with ProBond resin and cleaved with enterokinase; Invitrogen), to mention just two, can be employed according to the invention.

Once a particular recombinant DNA molecule is identified and isolated, several methods known in the art may be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As previously explained, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (*e.g.*, lambda), and plasmid and cosmid DNA vectors, to name but a few.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (*e.g.*, glycosylation, cleavage [*e.g.*, of signal sequence]) of proteins. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein

expressed. For example, expression in a bacterial system can be used to produce an nonglycosylated core protein product. However, the leptin binding protein expressed in bacteria may not be properly folded. Expression in yeast can produce a glycosylated product. Expression in eukaryotic cells can increase the likelihood of "native" glycosylation and folding of a heterologous protein an important criteria because the leptin binding protein is a glycosylated protein. Moreover, expression in mammalian cells can provide a tool for reconstituting, or constituting, leptin binding protein activity. Furthermore, different vector/host expression systems may affect processing reactions, such as proteolytic cleavages, to a different extent. Such a proteolytic cleavage is a necessary step in the formation of an embodiment of the present invention, *i.e.*, apolipoprotein J.

Vectors are introduced into the desired host cells by methods known in the art, *e.g.*, transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, lipofection (lysosome fusion), use of a gene gun, or a DNA vector transporter (see, *e.g.*, Wu et al., 1992, J. Biol. Chem. 267:963-967; Wu and Wu, 1988, J. Biol. Chem. 263:14621-14624; Hartmut et al., Canadian Patent Application No. 2,012,311, filed March 15, 1990).

A recombinant marker protein expressed as an integral membrane protein can be isolated and purified by standard methods. Generally, the integral membrane protein can be obtained by lysing the membrane with detergents, such as but not limited to, sodium dodecyl sulfate (SDS), Triton X-100, nonidet P-40 (NP-40), digoxin, sodium deoxycholate, and the like, including mixtures thereof. Solubilization can be enhanced by sonication of the suspension. Soluble forms of the protein can be obtained by collecting culture fluid, or solubilizing inclusion bodies, *e.g.*, by treatment with detergent, and if desired sonication or other mechanical processes, as described above. The solubilized or soluble protein can be isolated using various techniques, such as polyacrylamide gel electrophoresis (PAGE), isoelectric focusing, 2-dimensional gel electrophoresis, chromatography (*e.g.*, ion exchange, affinity,

immunoaffinity, and sizing column chromatography), centrifugation, differential solubility, immunoprecipitation, or by any other standard technique for the purification of proteins.

Non-coding Nucleic Acids

The present invention extends to the preparation of antisense nucleotides and ribozymes that may be used to interfere with the expression of the leptin binding protein at the translational level. This approach utilizes antisense nucleic acid and ribozymes to block translation of a specific mRNA, either by masking that mRNA with an antisense nucleic acid or cleaving it with a ribozyme.

Antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific mRNA molecule [*See* Weintraub, *Sci. Am.*, **262**:40-46 (1990); Marcus-Sekura, *Anal. Biochem.*, **172**:289-295 (1988)]. In the cell, they hybridize to that mRNA, forming a double-stranded molecule. The cell does not translate an mRNA complexed in this double-stranded form. Therefore, antisense nucleic acids interfere with the expression of mRNA into protein. Oligomers of about fifteen nucleotides and molecules that hybridize to the AUG initiation codon will be particularly efficient, since they are easy to synthesize and are likely to pose fewer problems than larger molecules when introducing them into weight modulator peptide-producing cells. Antisense methods have been used to inhibit the expression of many genes *in vitro* [(Marcus-Sekura, 1988 *supra*; Hambor *et al.*, *J. Exp. Med.*, **168**:1237-1245 (1988)].

Ribozymes are RNA molecules possessing the ability to specifically cleave other single-stranded RNA molecules in a manner somewhat analogous to DNA restriction endonucleases. Ribozymes were discovered from the observation that certain mRNAs have the ability to excise their own introns. By modifying the nucleotide sequence of these RNAs, researchers have been able to engineer molecules that recognize specific nucleotide sequences in an RNA molecule and cleave it [Cech, *J. Am. Med. Assoc.*,

260:3030-3034 (1988)]. Because they are sequence-specific, only mRNAs with particular sequences are inactivated.

Investigators have identified two types of ribozymes, *Tetrahymena*-type and "hammerhead"-type. *Tetrahymena*-type ribozymes recognize four-base sequences, while "hammerhead"-type recognize eleven- to eighteen-base sequences. The longer the recognition sequence, the more likely it is to occur exclusively in the target mRNA species. Therefore, hammerhead-type ribozymes are preferable to *Tetrahymena*-type ribozymes for inactivating a specific mRNA species, and eighteen base recognition sequences are preferable to shorter recognition sequences.

The DNA sequences for apolipoprotein J (*see* Kirszbaum, et al., *EMBO J.*, **8** 711-718 (1989) and Jordan-Starck, et al., *Journal of Lipid Research*, **35** 194-210 (1994)) may thus be used to prepare antisense molecules against and ribozymes that cleave mRNAs for the leptin binding protein, thus inhibiting its expression, and negating its effect on body weight.

Antibodies to the Leptin Binding Protein

According to the invention, the (L-LBP) complex (*e.g.*, the (L-aJ) complex) as isolated from natural sources, produced with naturally isolated proteins, recombinant proteins, or by chemical synthesis, or mixtures thereof (such as a naturally isolated leptin binding protein bound to a chemically synthesized leptin) or antigenic fragments or other derivatives or analogs thereof, may be used as an immunogen to generate antibodies that recognize the (L-LBP) complex. Similarly the leptin binding protein (*e.g.*, apolipoprotein J) as isolated from natural sources, or the recombinant protein, or by chemical synthesized protein, or antigenic fragments or other derivatives or analogs thereof, may be used as an immunogen to generate antibodies that recognize the leptin binding protein. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library.

A molecule is "antigenic" when it is capable of specifically interacting with an antigen recognition molecule of the immune system, such as an immunoglobulin (antibody) or T cell antigen receptor. An antigenic polypeptide contains at least about 5, and preferably at least about 10, amino acids. An antigenic portion of a molecule can be that portion that is immunodominant for antibody or T cell receptor recognition, or it can be a portion used to generate an antibody to the molecule by conjugating the antigenic portion to a carrier molecule for immunization. A molecule that is antigenic need not be itself immunogenic, *i.e.*, capable of eliciting an immune response without a carrier.

An "antibody" is any immunoglobulin, including antibodies and fragments thereof, that binds a specific epitope. The term encompasses polyclonal, monoclonal, and chimeric antibodies, the last mentioned described in further detail in U.S. Patent Nos. 4,816,397 and 4,816,567, as well as antigen binding portions of antibodies, including Fab, $F(ab')_2$ and F(v) (including single chain antibodies). Accordingly, the phrase "antibody molecule" in its various grammatical forms as used herein contemplates both an intact immunoglobulin molecule and an immunologically active portion of an immunoglobulin molecule containing the antibody combining site. An "antibody combining site" is that structural portion of an antibody molecule comprised of heavy and light chain variable and hypervariable regions that specifically binds antigen.

Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contains the paratope, including those portions known in the art as Fab, Fab', $F(ab')_2$ and F(v), which portions are preferred for use in the therapeutic methods described herein.

Fab and $F(ab')_2$ portions of antibody molecules are prepared by the proteolytic reaction of papain and pepsin, respectively, on substantially intact antibody molecules by methods that are well-known. See, for example, U.S. Patent No. 4,342,566 to

Theofilopolous *et al.* Fab' antibody molecule portions are also well-known and are produced from $F(ab')_2$ portions followed by reduction of the disulfide bonds linking the two heavy chain portions as with mercaptoethanol, and followed by alkylation of the resulting protein mercaptan with a reagent such as iodoacetamide. An antibody containing intact antibody molecules is preferred herein.

The phrase "monoclonal antibody" in its various grammatical forms refers to an antibody having only one species of antibody combining site capable of immunoreacting with a particular antigen. A monoclonal antibody thus typically displays a single binding affinity for any antigen with which it immunoreacts. A monoclonal antibody may therefore contain an antibody molecule having a plurality of antibody combining sites, each immunospecific for a different antigen; e.g., a bispecific (chimeric) monoclonal antibody.

The term "adjuvant" refers to a compound or mixture that enhances the immune response to an antigen. An adjuvant can serve as a tissue depot that slowly releases the antigen and also as a lymphoid system activator that non-specifically enhances the immune response [Hood *et al.*, in *Immunology*, p. 384, Second Ed., Benjamin/Cummings, Menlo Park, California (1984)]. Often, a primary challenge with an antigen alone, in the absence of an adjuvant, will fail to elicit a humoral or cellular immune response. Adjuvants include, but are not limited to, complete Freund's adjuvant, incomplete Freund's adjuvant, saponin, mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil or hydrocarbon emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (*bacille Calmette-Guerin*) and *Corynebacterium parvum*. Preferably, the adjuvant is pharmaceutically acceptable.

Various procedures known in the art may be used for the production of polyclonal antibodies to the leptin binding protein or the (L-LBP) complex, or fragment,

derivative or analog thereof. For the production of antibody, various host animals can be immunized by injection with the appropriate antigen, or a derivative (*e.g.*, fragment or fusion protein) thereof, including but not limited to rabbits, mice, rats, sheep, goats, etc. In one embodiment, the antigen can be conjugated to an immunogenic carrier, *e.g.*, bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH). Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (*bacille Calmette-Guerin*) and *Corynebacterium parvum*.

For preparation of monoclonal antibodies directed toward the leptin binding protein or the (L-LBP) complex or fragments, analogs, or derivatives thereof, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used. These include but are not limited to the hybridoma technique originally developed by Kohler *et al.*, *Nature*, **256**:495-497 (1975), as well as the trioma technique, the human B-cell hybridoma technique [Kozbor *et al.*, *Immunology Today*, 4:72 (1983)], and the EBV-hybridoma technique to produce human monoclonal antibodies [Cole *et al.*, in *Monoclonal Antibodies and Cancer Therapy*, pp. 77-96, Alan R. Liss, Inc., (1985)]. Immortal, antibody-producing cell lines can be created by techniques other than fusion, such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. *See, e.g.*, M. Schreier *et al.*, "Hybridoma Techniques" (1980); Hammerling *et al.*, "Monoclonal Antibodies And T-cell Hybridomas" (1981); Kennett *et al.*, "Monoclonal Antibodies" (1980); *see also* U.S. Patent Nos. 4,341,761; 4,399,121; 4,427,783; 4,444,887; 4,451,570; 4,466,917; 4,472,500; 4,491,632; and 4,493,890.

In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing recent technology (PCT/US90/02545). According to

the invention, human antibodies may be used and can be obtained by using human hybridomas [Cote *et al.*, *Proc. Natl. Acad. Sci. USA*, **80**:2026-2030 (1983)] or by transforming human B cells with EBV virus *in vitro* (Cole *et al.*, 1985, *supra*). In fact, according to the invention, techniques developed for the production of "chimeric antibodies" [Morrison *et al.*, *J. Bacteriol.*, 159-870 (1984); Neuberger *et al.*, *Nature*, **312**:604-608 (1984); Takeda *et al.*, *Nature*, **314**:452-454 (1985)] by splicing the genes from a mouse antibody molecule specific for a specific leptin binding protein or (L-LBP) complex together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention. Such human or humanized chimeric antibodies are preferred for use in therapy of human diseases or disorders (described *infra*), since the human or humanized antibodies are much less likely than xenogenic antibodies to induce an immune response, in particular an allergic response, themselves.

According to the invention, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce leptin binding protein or (L-LBP) complex-specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries [Huse *et al.*, *Science*, **246**:1275-1281 (1989)] to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for the leptin binding protein or the (L-LBP) complex, or its derivatives, or analogs.

Antibody fragments which contain the idiotype of the antibody molecule can be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule: the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, *e.g.*, radioimmunoassay, ELISA (enzyme-linked immunosorbent assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), Western blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc. In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention. For example, to select antibodies which recognize a specific epitope of the leptin binding protein, one may assay generated hybridomas for a product which bind to the leptin binding protein fragment containing such a epitope. For selection of an antibody specific for the (L-LBP) complex, for example, from a particular species of animal, one can select such a antibody on the basis of positive binding with the (L-LBP) complex isolated from cells of that same species of animal.

The foregoing antibodies can be used in methods known in the art relating to the localization and the activity of the leptin binding protein, or the (L-LBP) complex *e.g.*, for Western blotting, imaging these antigens *in situ*, measuring levels thereof in appropriate physiological samples, etc.

In a specific embodiment, antibodies that agonize or antagonize the activity of the leptin binding protein can be generated. Such antibodies can be tested using the assays described *infra*.

In a specific embodiment, antibodies are developed by immunizing rabbits with synthetic peptides predicted by the protein sequence or with recombinant proteins made using bacterial expression vectors. The choice of synthetic peptides is made after careful analysis of the predicted protein structure, as described above. In particular, peptide sequences between putative cleavage sites are chosen. Synthetic peptides are conjugated to a carrier such as KLH hemocyanin or BSA using carbodiimide and used in Freund's adjuvant to immunize rabbits. In order to prepare recombinant protein, the pGEX vector can be used to express the polypeptide (Smith *et al.*, 1988, *supra*). Alternatively, one can use only hydrophilic domains to generate the fusion protein. The expressed protein will be prepared in quantity and used to immunize rabbits in Freund's adjuvant.

In another specific embodiment, leptin binding protein is used to immunize chickens, and the chicken anti-leptin binding protein antibodies are recovered from egg yolk, *e.g.*, by affinity purification on an leptin binding protein affinity column. In a related embodiment, the (L-LBP) complex is used to immunize the chickens. Preferably, chickens used in immunization are kept under specific pathogen free (SPF) conditions.

In yet another embodiment, leptin binding protein is used to immunize rabbits, and the polyclonal antibodies are immunopurified prior to further use. The purified antibodies are particularly useful for semi-quantitative assays, particularly for detecting the presence of circulating leptin binding protein in serum or plasma. In a related embodiment the (L-LBP) complex is used to immunize the rabbits. In a specific embodiment, the (L-LBP) complex is a (L-aJ) complex.

Panels of monoclonal antibodies produced against the leptin binding protein can be screened for preferential affinity for the (L-LBP) complex relative to the leptin binding protein not bound to leptin. These antibodies are of particular interest. Such monoclonals can be readily identified in assays comparing total leptin binding protein

versus (L-LBP) complex in conjunction with Western analysis, for example. High affinity antibodies of this type are also useful when immunoaffinity purification of the (L-LBP) complex is required.

Preferably, the anti-leptin binding protein antibody used in the diagnostic and therapeutic methods of this invention is an affinity-purified polyclonal antibody. More preferably, the antibody is a monoclonal antibody (mAb). In addition, it is preferable for the anti-modulator antibody molecules used herein be in the form of Fab, Fab', $F(ab')_2$ or F(v) portions of whole antibody molecules.

In some cases raising the antibodies directly against the (L-LBP) complex, as described above, will require that the leptin binding protein and leptin in the (L-LBP) complex be chemically cross-linked. The leptin binding protein and leptin may be crosslinked, through a polyfunctional molecule, *i.e.*, a polyfunctional cross-linker. As used herein, the term "polyfunctional molecule" encompasses molecules having one functional group that can react more than one time in succession, such as formaldehyde (although formaldehyde is not indicated for use due to its potential carcinogenicity), as well as molecules with more than one reactive group. As used herein, the term "reactive group" refers to a functional group on the cross-linker that reacts with a functional group on the protein so as to form a covalent bond between the cross-linker and that protein. The term "functional group" retains its standard meaning in organic chemistry. The polyfunctional molecules which can be used are can be biocompatible linkers, *i.e.*, they are non-carcinogenic, nontoxic, and substantially non-immunogenic *in vivo*. Polyfunctional cross-linkers such as those known in the art and described herein can be readily tested in animal models to determine their biocompatibility. The polyfunctional molecule is preferably bifunctional. As used herein, the term "bifunctional molecule" refers to a molecule with two reactive groups. The bifunctional molecule may be hetero-bifunctional or homo-bifunctional. A hetero-bifunctional cross-linker allows for vectorial conjugation. It is preferred that the polyfunctional molecule to be sufficiently soluble

in water for the cross-linking reactions to occur in aqueous solutions such as in aqueous solutions buffered at pH 6 to 8 an for the resulting conjugate to remain water soluble. Typically, the polyfunctional molecule covalently bonds with an amino or a sulfhydryl functional group. However, polyfunctional molecules reactive with other functional groups, such as carboxylic acids or hydroxyl groups may be of use.

Diagnostic Implications

The present invention also relates to a variety of diagnostic applications, including methods for detecting the presence of conditions and/or stimuli that impact upon abnormalities in endogenous leptin pathway, by reference to their ability to elicit the activities which are mediated by the leptin binding protein alone or in conjunction with leptin. As mentioned earlier, the leptin binding protein and the (L-LBP) complex can be used to produce antibodies to themselves by a variety of known techniques, and such antibodies could then be isolated and utilized as in tests for the presence of particular transcriptional activity in suspect target cells.

In one such method of diagnosing physiological obesity in a human according to the present invention, a mammalian subject is diagnosed with physiological obesity. First, the amount of leptin bound to the leptin binding protein in a test sample taken from the blood of the subject human is determined. Next, the amount is compared with a range of values for humans without physiological obesity. A determined amount less than the known range of values indicates a diagnosis of physiological obesity.

In another method of diagnosing physiological obesity in a humanaccording to the present invention, a mammalian subject is diagnosed with physiological obesity. First, the amount of leptin bound to the leptin binding protein in a test sample taken from the blood of the subject human is determined. Next, the amount of leptin that is not bound to the leptin binding protein in the test sample is determined. Then, the ratio of bound leptin to unbound leptin is calculated. Next, the ratio is compared with a range of values for the ratio in humans without physiological obesity. A determined

amount less than the known range of values indicates a diagnosis of physiological obesity.

Antibody-based Diagnostics

As suggested earlier, a diagnostic method useful in the present invention comprises examining a cellular sample or medium by means of an assay including an effective amount of an antagonist to the leptin binding protein and/or the (L-LBP) complex, such as an anti-leptin binding protein antibody or an anti-(L-LBP) complex antibody, preferably an affinity-purified polyclonal antibody, and more preferably a mAb. In addition, it is preferable for the anti-leptin binding protein or (L-LBP) complex antibody molecules used herein be in the form of Fab, Fab', $F(ab')_2$ or F(v) portions or whole antibody molecules. As previously discussed, patients capable of benefiting from this method include those suffering from cancer, AIDS, obesity or other conditions where abnormal body weight is a characteristic or factor. Methods for isolating the anti-leptin binding protein and anti-(L-LBP)complex antibodies and for determining and optimizing the ability of these antibodies to assist in the examination of the target cells are all well-known in the art.

Also, antibodies including both polyclonal and monoclonal antibodies, and drugs that modulate the production or activity of the leptin binding protein and other recognition factors and/or their subunits may possess certain diagnostic applications and may for example, be utilized for the purpose of detecting and/or measuring conditions where abnormalities in endogenous leptin pathway are or may be likely to develop. For example, the leptin binding protein or the (L-LBP) complex or their active fragments may be used to produce both polyclonal and monoclonal antibodies to themselves in a variety of cellular media, by known techniques, such as the hybridoma technique utilizing, for example, fused mouse spleen lymphocytes and myeloma cells. These techniques are described in detail below.

The presence of the leptin binding protein or the (L-LBP) complex in cells can be ascertained by the usual immunological procedures applicable to such determinations. A number of useful procedures are known. Procedures which are especially useful utilize either the leptin binding protein or the (L-LBP) complex labeled with a detectable label, antibody Ab_1 labeled with a detectable label, or antibody Ab_2 labeled with a detectable label.

The procedures and their application are all familiar to those skilled in the art and accordingly may be utilized within the scope of the present invention. A "competitive" procedure is described in U.S. Patent Nos. 3,654,090 and 3,850,752. A "sandwich" procedure is described in U.S. Patent Nos. RE 31,006 and 4,016,043. Still other procedures are known such as the "double antibody", or "DASP" procedure.

In each instance, the leptin binding protein or the (L-LBP) complex bind with one or more antibody(ies) or binding partners and one member of the complex is labeled with a detectable label. The fact that a complex has formed and, if desired, the amount thereof, can be determined by known methods applicable to the detection of labels.

The labels most commonly employed for these studies are radioactive elements, enzymes, chemicals which fluoresce when exposed to ultraviolet light, and others. Labels also include elements that can be reorganized by another binding molecule, such as biotin. Antibody or antigen can also be labeled by creating a phosphorylation site in the sequence [see European Patent No. 0372707 by Sidney Pestka], *e.g.*, by using plasmid pGEX-TTK (Pharmacia, Product No. 27-45-8701).

A number of fluorescent materials are known and can be utilized as labels. These include, for example, fluorescein, rhodamine and auramine. A particular detecting

material is anti-rabbit antibody prepared in goats and conjugated with fluorescein through an isothiocyanate.

The leptin binding protein and the (L-LBP) complex or their binding partners can also be labeled with a radioactive element or with an enzyme. The radioactive label can be detected by any of the currently available counting procedures. The preferred isotope may be selected from ^{3}H, ^{14}C, ^{32}P, ^{35}S, ^{36}Cl, ^{51}Cr, ^{57}Co, ^{58}Co, ^{59}Fe, ^{90}Y, ^{125}I, ^{131}I, and ^{186}Re.

Enzyme labels are likewise useful, and can be detected by any of the presently utilized colorimetric, spectrophotometric, fluorospectrophotometric, amperometric or gasometric techniques. The enzyme is conjugated to the selected particle by reaction with bridging molecules such as carbodiimides, diisocyanates, glutaraldehyde and the like. Many enzymes which can be used in these procedures are known and can be utilized. The preferred enzymes are peroxidase, ß-glucuronidase, ß-D-glucosidase, ß-D-galactosidase, urease, glucose oxidase plus peroxidase and alkaline phosphatase. U.S. Patent Nos. 3,654,090; 3,850,752; and 4,016,043 are referred to by way of example for their disclosure of alternate labeling material and methods.

In a further embodiment of this invention, certain test kits are suitable. In accordance with the testing techniques discussed above, one class of such kits will contain at least the labeled leptin binding protein or the labeled (L-LBP) complex, for instance an antibody specific thereto, and directions, of course, depending upon the method selected, e.g., "competitive," "sandwich," "DASP" and the like. The kits may also contain peripheral reagents such as buffers, stabilizers, etc.

Nucleic Acid and Protein Based Diagnostics

The mRNAs that encode the leptin binding proteins may be used as a diagnostic for determining abnormalities in the endogenous leptin pathway. Methods of measuring the mRNA, such as performing Northern blots are well known to those of skill in the

art. Similarly, searching for mutations in a gene encoding the leptin binding protein may performed using standard RFLP analysis. The L-LBP complex may be monitored by Western blots or through immunoprecipitation with a an antibody to leptin, an antibody to the leptin binding protein or an antibody to the L-LBP complex itself. Confirmation of the identity of the immunoprecipitate may be ascertained by performing SDS-PAGE on the immunoprecipitate under either reducing and/or non-reducing conditions.

Therapeutic Implications

The leptin binding protein alone or in conjunction with leptin, nucleic acids that encode the leptin binding protein, and antibodies to it as well as to the (L-LBP) complex have significant therapeutic potential. Preferably, a therapeutically effective amount of such an agent is administered in a pharmaceutically acceptable carrier, diluent, or excipient.

The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similarly untoward reaction, such as gastric upset, dizziness and the like, when administered to a human. Preferably, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the federal or a state government or listed in the *U.S. Pharmacopeia* or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in Martin, *Remington's Pharmaceutical Sciences*, 18th Ed., Mack Publishing Co., Easton, PA, (1990).

The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to reduce by at least about 15%, preferably by at least 50%, more preferably by at least 90%, and most preferably prevent, a clinically significant deficit in the activity, function and response of the host. Alternatively, a therapeutically effective amount is sufficient to cause an improvement in a clinically significant condition in the host.

Administration of the leptin binding protein alone or in conjunction with leptin, including a complex thereof (*i.e.* the (L-LBP) complex), results in an improvement of an abnormality in the endogenous leptin pathway. The leptin binding protein and the (L-LBP) complex can be prepared by isolation from natural sources (*e.g.*, purified from plasma or serum, as described in detail, herein) or using recombinant proteins obtained by standard bacterial and/or mammalian expression vectors, synthetically. Alternatively, increased expression of the native leptin binding protein may be induced by homologous recombination techniques, as described *supra*.

Reduction of the leptin binding protein (by developing antagonists, inhibitors, use of neutralizing antibodies, or antisense molecules) should result in weight gain as might be desirable for the treatment of the weight loss associated with cancer, AIDS or anorexia nervosa. Modulation of the leptin binding protein can be useful for reducing body weight (by increasing its activity) or increasing body weight (by decreasing its activity).

Polypeptide-based Therapeutic Treatment

In the simplest analysis, a *leptin binding protein* gene effects body weight in mammals, in particular, mice and man through its product's interaction with leptin. This gene product, and, correspondingly, cognate molecules, are part of the endogenous leptin pathway that effect how adipose tissue communicates with the brain and the other organs.

The leptin binding protein (*e.g.*, apolipoprotein J), or functionally active fragment thereof, or an antagonist thereof, alone or in conjunction with leptin including in a complex thereof, can be administered orally or parenterally, preferably parenterally. Because metabolic homeostasis is a continuous process, controlled release administration of these agents are preferred. For example, the polypeptide(s) may be administered using intravenous infusion, an implantable osmotic pump, a trans-dermal patch, liposomes, or other modes of administration. In one embodiment, a pump may be used [*Langer et al.*, eds., *Medical Applications of Controlled Release*, CRC Pres., Boca Raton, Florida (1974); Sefton, *CRC Crit. Ref. Biomed. Eng.*, **14**:201 (1987); Buchwald *et al.*, *Surgery*, **88**:507 (1980); Saudek *et al.*, *N. Engl. J. Med.*, **321**:574 (1989)]. In another embodiment, polymeric materials can be used [Langer, 1974, *supra*; Sefton, 1987, *supra*; Smolen *et al.*, eds., *Controlled Drug Bioavailability, Drug Product Design and Performance*, Wiley, New York (1984); Ranger *et al.*, *J. Macromol. Sci. Rev. Macromol. Chem.*, **23**:61 (1983); *see* also Levy *et al.*, *Science*, **228**:190 (1985); During *et al.*, *Ann. Neurol.*, **25**:351 (1989); Howard *et al.*, *J. Neurosurg.*, **71**:105 (1989)]. In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, *i.e.*, the brain, thus requiring only a fraction of the systemic dose [*see, e.g.*, Goodson, in *Medical Applications of Controlled Release*, vol. 2, pp. 115-138 (1984)]. Other controlled release systems are discussed in the review by Langer, *Science*, **249**:1527-1533 (1990). In another embodiment, the therapeutic compound can be delivered in a vesicle, in particular a liposome (*see* Langer, 1990 *supra*); Treat *et al.*, in *Liposomes in the Therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, *ibid.*, pp. 317-327; *see generally ibid.*)

In a further aspect, recombinant cells that have been transformed with the *leptin binding protein* gene and that express high levels of the polypeptide can be transplanted in a subject in need of leptin binding protein. Preferably autologous cells transformed with the *leptin binding protein* gene are transplanted to avoid rejection;

alternatively, technology is available to shield non-autologous cells that produce soluble factors within a polymer matrix that prevents immune recognition and rejection.

The leptin binding protein alone or in conjunction with leptin including a complex thereof, can be delivered by intravenous, intraarterial, intraperitoneal, intramuscular, or subcutaneous routes of administration. Alternatively, these agents, properly formulated, can be administered by nasal or oral administration. A constant supply of these agents can be ensured by providing a therapeutically effective dose (*i.e.*, a dose effective to induce metabolic changes in a subject) at the necessary intervals, *e.g.*, daily, every 12 hours, etc. These parameters will depend on the severity of the condition being treated, other actions, such as diet modification, that are implemented, the weight, age, and sex of the subject, and other criteria, which can be readily determined according to standard good medical practice by those of skill in the art.

Pharmaceutical Compositions

In yet another aspect of the present invention, provided are pharmaceutical compositions of the above. Such pharmaceutical compositions may be for administration for injection, or for oral, pulmonary, nasal or other forms of administration. In general, comprehended by the invention are pharmaceutical compositions comprising effective amounts of protein(s) or derivative products of the invention together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content (*e.g.*, Tris-HCl, acetate, phosphate), pH and ionic strength; additives such as detergents and solubilizing agents (e.g., Tween 80, Polysorbate 80), anti-oxidants (*e.g.*, ascorbic acid, sodium metabisulfite), preservatives (*e.g.*, Thimersol, benzyl alcohol) and bulking substances (*e.g.*, lactose, mannitol); incorporation of the material into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into liposomes. Hyaluronic acid may also be used. Such compositions may influence the physical

state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the leptin binding protein and derivatives. *See, e.g., Martin, Remington's Pharmaceutical Sciences*, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages 1435-1712 which are herein incorporated by reference. The compositions may be prepared in liquid form, or may be in dried powder, such as lyophilized form.

Oral Delivery. Contemplated for use herein are oral solid dosage forms, which are described generally in *Martin, Remington's Pharmaceutical Sciences*, 18th Ed. (1990 Mack Publishing Co. Easton PA 18042) at Chapter 89, which is herein incorporated by reference. Solid dosage forms include tablets, capsules, pills, troches or lozenges, cachets or pellets. Also, liposomal or proteinoid encapsulation may be used to formulate the present compositions (as, for example, proteinoid microspheres reported in U.S. Patent No. 4,925,673). Liposomal encapsulation may be used and the liposomes may be derivatized with various polymers (*E.g.*, U.S. Patent No. 5,013,556). A description of possible solid dosage forms for the therapeutic is given by Marshall, in *Modern Pharmaceutics*, Chapter 10, Banker and Rhodes ed., (1979), herein incorporated by reference. In general, the formulation will include the protein (or chemically modified protein), and inert ingredients which allow for protection against the stomach environment, and release of the biologically active material in the intestine.

Also specifically contemplated are oral dosage forms of the above derivatized proteins. The protein(s) may be chemically modified so that oral delivery of the derivative is efficacious. Generally, the chemical modification contemplated is the attachment of at least one moiety to the protein (or peptide) molecule itself, where said moiety permits (a) inhibition of proteolysis; and (b) uptake into the blood stream from the stomach or intestine. Also desired is the increase in overall stability of the protein and increase in circulation time in the body. Examples of such moieties include: polyethylene glycol, copolymers of ethylene glycol and propylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone and

polyproline. Abuchowski *et al.*, 1981, *supra*; Newmark *et al.*, *J. Appl. Biochem.*, 4:185-189 (1982). Other polymers that could be used are poly-1,3-dioxolane and poly-1,3,6-tioxocane. Preferred for pharmaceutical usage, as indicated above, are polyethylene glycol moieties.

For the protein(s) (or derivative(s)) the location of release may be the stomach, the small intestine (the duodenum, the jejunum, or the ileum), or the large intestine. One skilled in the art has available formulations which will not dissolve in the stomach, yet will release the material in the duodenum or elsewhere in the intestine. Preferably, the release will avoid the deleterious effects of the stomach environment, either by protection of the protein (or derivative) or by release of the biologically active material beyond the stomach environment, such as in the intestine.

To ensure full gastric resistance, a coating impermeable to at least pH 5.0 is essential. Examples of the more common inert ingredients that are used as enteric coatings are cellulose acetate trimellitate (CAT), hydroxypropylmethylcellulose phthalate (HPMCP), HPMCP 50, HPMCP 55, polyvinyl acetate phthalate (PVAP), Eudragit L30D, Aquateric, cellulose acetate phthalate (CAP), Eudragit L, Eudragit S, and Shellac. These coatings may be used as mixed films.

A coating or mixture of coatings can also be used on tablets, which are not intended for protection against the stomach. This can include sugar coatings, or coatings which make the tablet easier to swallow. Capsules may consist of a hard shell (such as gelatin) for delivery of dry therapeutic *i.e.* powder; for liquid forms, a soft gelatin shell may be used. The shell material of cachets could be thick starch or other edible paper. For pills, lozenges, molded tablets or tablet triturates, moist massing techniques can be used.

The therapeutic can be included in the formulation as fine multiparticulates in the form of granules or pellets of particle size about one millimeter. The formulation of

the material for capsule administration could also be as a powder, lightly compressed plugs or even as tablets. The therapeutic could be prepared by compression.

Colorants and flavoring agents may all be included. For example, the protein(s) (or derivative(s)) may be formulated (such as by liposome or microsphere encapsulation) and then further contained within an edible product, such as a refrigerated beverage containing colorants and flavoring agents.

One may dilute or increase the volume of the therapeutic with an inert material. These diluents could include carbohydrates, especially mannitol, α-lactose, anhydrous lactose, cellulose, sucrose, modified dextrans and starch. Certain inorganic salts may be also be used as fillers including calcium triphosphate, magnesium carbonate and sodium chloride. Some commercially available diluents are Fast-Flo, Emdex, STA-Rx 1500, Emcompress and Avicell.

Disintegrants may be included in the formulation of the therapeutic into a solid dosage form. Materials used as disintegrants include but are not limited to starch including the commercial disintegrant based on starch, Explotab. Sodium starch glycolate, Amberlite, sodium carboxymethylcellulose, ultramylopectin, sodium alginate, gelatin, orange peel, acid carboxymethyl cellulose, natural sponge and bentonite may all be used. Another form of the disintegrants are the insoluble cationic exchange resins. Powdered gums may be used as disintegrants and as binders and these can include powdered gums such as agar, Karaya or tragacanth. Alginic acid and its sodium salt are also useful as disintegrants.

Binders may be used to hold the therapeutic agent together to form a hard tablet and include materials from natural products such as acacia, tragacanth, starch and gelatin. Others include methyl cellulose (MC), ethyl cellulose (EC) and carboxymethyl cellulose (CMC). Polyvinyl pyrrolidone (PVP) and hydroxypropylmethyl cellulose (HPMC) could both be used in alcoholic solutions to granulate the therapeutic.

An anti-frictional agent may be included in the formulation of the therapeutic to prevent sticking during the formulation process. Lubricants may be used as a layer between the therapeutic and the die wall, and these can include but are not limited to: stearic acid including its magnesium and calcium salts, polytetrafluoroethylene (PTFE), liquid paraffin, vegetable oils and waxes. Soluble lubricants may also be used such as sodium lauryl sulfate, magnesium lauryl sulfate, polyethylene glycol of various molecular weights, and Carbowax 4000 and 6000.

Glidants that might improve the flow properties of the drug during formulation and to aid rearrangement during compression might be added. The glidants may include starch, talc, pyrogenic silica and hydrated silicoaluminate.

To aid dissolution of the therapeutic into the aqueous environment, a surfactant might be added as a wetting agent. Surfactants may include anionic detergents such as sodium lauryl sulfate, dioctyl sodium sulfosuccinate and dioctyl sodium sulfonate. Cationic detergents might be used and could include benzalkonium chloride or benzethomium chloride. The list of potential nonionic detergents that could be included in the formulation as surfactants are lauromacrogol 400, polyoxyl 40 stearate, polyoxyethylene hydrogenated castor oil 10, 50 and 60, glycerol monostearate, polysorbate 40, 60, 65 and 80, sucrose fatty acid ester, methyl cellulose and carboxymethyl cellulose. These surfactants could be present in the formulation of the protein or derivative either alone or as a mixture in different ratios.

Additives which potentially enhance uptake of the protein(s) (or derivative(s)) are for instance the fatty acids oleic acid, linoleic acid and linolenic acid.

Controlled release formulation may be desirable. The drug could be incorporated into an inert matrix which permits release by either diffusion or leaching mechanisms *i.e.*, gums. Slowly degenerating matrices may also be incorporated into the formulation.

Another form of a controlled release of this therapeutic is by a method based on the Oros therapeutic system (Alza Corp.), i.e. the drug is enclosed in a semipermeable membrane which allows water to enter and push drug out through a single small opening due to osmotic effects. Some enteric coatings also have a delayed release effect.

Other coatings may be used for the formulation. These include a variety of sugars which could be applied in a coating pan. The therapeutic agent could also be given in a film-coated tablet; the materials used in this instance are divided into 2 groups. The first are the nonenteric materials and include methyl cellulose, ethyl cellulose, hydroxyethyl cellulose, methylhydroxy-ethyl cellulose, hydroxypropyl cellulose, hydroxypropyl-methyl cellulose, sodium carboxy-methyl cellulose, providone and the polyethylene glycols. The second group consists of the enteric materials that are commonly esters of phthalic acid.

A mix of materials might be used to provide the optimum film coating. Film coating may be carried out in a pan coater or in a fluidized bed or by compression coating.

Pulmonary Delivery. Also contemplated herein is pulmonary delivery of the leptin binding protein alone or in conjunction with leptin including a complex thereof (or derivatives thereof). The protein(s) (or derivative(s)) are delivered to the lungs of a mammal while inhaling and traverse across the lung epithelial lining to the blood-stream. Other reports of this include Adjei *et al.*, *Pharmaceutical Research*, **7(6)**:565-569 (1990); Adjei *et al.*, *International Journal of Pharmaceutics*, **63**:135-144 (1990) (leuprolide acetate); Braquet *et al.*, *Journal of Cardiovascular Pharmacology*, **13**(suppl. 5):143-146 (1989) (endothelin-1); Hubbard *et al.*, *Annals of Internal Medicine*, **3(3)**:206-212 (1989) (α1- antitrypsin); Smith *et al.*, *J. Clin. Invest.*, **84**:1145-1146 (1989) (α1-proteinase); Oswein *et al.*, "Aerosolization of Proteins", *Proceedings of Symposium on Respiratory Drug Delivery II*, Keystone, Colorado, (March 1990) (recombinant human growth hormone); Debs *et al.*, *J. Immunol.*,

140:3482-3488 (1988) and Platz *et al.*, U.S. Patent No. 5,284,656 (granulocyte colony stimulating factor). Contemplated for use in the practice of this invention are a wide range of mechanical devices designed for pulmonary delivery of therapeutic products, including but not limited to nebulizers, metered-dose inhalers, and powder inhalers, all of which are familiar to those skilled in the art.

Some specific examples of commercially available devices suitable for the practice of this invention are the Ultravent nebulizer, manufactured by Mallinckrodt, Inc., St. Louis, Missouri; the Acorn II nebulizer, manufactured by Marquest Medical Products, Englewood, Colorado; the Ventolin metered dose inhaler, manufactured by Glaxo Inc., Research Triangle Park, North Carolina; and the Spinhaler powder inhaler, manufactured by Fisons Corp., Bedford, Massachusetts.

All such devices require the use of formulations suitable for the dispensing of protein(s) (or derivative(s)). Typically, each formulation is specific to the type of device employed and may involve the use of an appropriate propellant material, in addition to the usual diluents, adjuvants and/or carriers useful in therapy. Also, the use of liposomes, microcapsules or microspheres, inclusion complexes, or other types of carriers is contemplated. Chemically modified protein(s) may also be prepared in different formulations depending on the type of chemical modification or the type of device employed.

Formulations suitable for use with a nebulizer, either jet or ultrasonic, will typically comprise proteins (or derivative(s)) dissolved in water at a concentration of about 0.1 to 25 mg of biologically active protein or protein complex per ml of solution. The formulation may also include a buffer and a simple sugar (*e.g.*, for protein stabilization and regulation of osmotic pressure). The nebulizer formulation may also contain a surfactant, to reduce or prevent surface induced aggregation of the protein caused by atomization of the solution in forming the aerosol.

Formulations for use with a metered-dose inhaler device will generally comprise a finely divided powder containing the protein (or derivative) suspended in a propellant with the aid of a surfactant. The propellant may be any conventional material employed for this purpose, such as a chlorofluorocarbon, a hydrochlorofluorocarbon, a hydrofluorocarbon, or a hydrocarbon, including trichlorofluoromethane, dichlorodifluoromethane, dichlorotetrafluoroethanol, and 1,1,1,2-tetrafluoroethane, or combinations thereof. Suitable surfactants include sorbitan trioleate and soya lecithin. Oleic acid may also be useful as a surfactant.

Formulations for dispensing from a powder inhaler device will comprise a finely divided dry powder containing protein (or derivative) and may also include a bulking agent, such as lactose, sorbitol, sucrose, or mannitol in amounts which facilitate dispersal of the powder from the device, *e.g.*, 50 to 90% by weight of the formulation. The protein(s) (or derivative(s)) should most advantageously be prepared in particulate form with an average particle size of less than 10 μm (or microns), most preferably 0.5 to 5 μm, for most effective delivery to the distal lung.

Transmucosal Delivery. Transmucosal delivery, *e.g.*, pulmonary, nasal, buccal, oral-pharyngeal, rectal, and vaginal delivery, may be preferred where oral delivery is ineffective or impractical, to avoid parenteral injection, and to facilitate rapid uptake without first-pass metabolism in the liver. Accordingly, transmucosal delivery of the protein(s) (or derivative(s)) are also contemplated. Formulations for transmucosal delivery preferably include a mucosal penetration enhancer.

The term "mucosal penetration enhancer" refers to a reagent that increases the rate or facility of transmucosal penetration of ketamine, such as but not limited to, a bile salt, fatty acid, surfactant or alcohol. In specific embodiments, the permeation enhancer can be sodium cholate, sodium dodecyl sulphate, sodium deoxycholate, taurodeoxycholate, sodium glycocholate, dimethylsulfoxide or ethanol. Suitable penetration enhancers also include glycyrrhetinic acid (U.S. Patent No. 5,112,804 to

Kowarski) and polysorbate-80, the latter preferably in combination with an non-ionic surfactant such as nonoxynol-9, laureth-9, poloxamer-124, octoxynol-9, or lauramide-DEA (European Patent EP 0 242 643 B1 by Stoltz).

Transdermal delivery. In addition, the invention contemplates transdermal delivery of a pharmaceutical composition of the invention. Transdermal delivery advantageously provides for long term, steady drug delivery, which is particularly indicated for regulating metabolic homeostasis.

Various and numerous methods are known in the art for transdermal administration of a drug, *e.g.*, via a transdermal patch. Transdermal patches are described in for example, U.S. Patent No. 5,407,713, issued April 18, 1995 to Rolando et al.; U.S. Patent No. 5,352,456, issued October 4, 1004 to Fallon et al.; U.S. Patent No. 5,332,213 issued August 9, 1994 to D'Angelo et al.; U.S. Patent No. 5,336,168, issued August 9, 1994 to Sibalis; U.S. Patent No. 5,290,561, issued March 1, 1994 to Farhadieh et al.; U.S. Patent No. 5,254,346, issued October 19, 1993 to Tucker et al.; U.S. Patent No. 5,164,189, issued November 17, 1992 to Berger et al.; U.S. Patent No. 5,163,899, issued November 17, 1992 to Sibalis; U.S. Patent Nos. 5,088,977 and 5,087,240, both issued February 18, 1992 to Sibalis; U.S. Patent No. 5,008,110, issued April 16, 1991 to Benecke et al.; and U.S. Patent No. 4,921,475, issued May 1, 1990 to Sibalis, the disclosure of each of which is incorporated herein by reference in its entirety.

It can be readily appreciated that a transdermal route of administration may be enhanced by use of a dermal penetration enhancer, *e.g.*, such as enhancers described in U.S. Patent No. 5,164,189 (*supra*), U.S. Patent No. 5,008,110 (*supra*), and U.S. Patent No. 4,879,119, issued November 7, 1989 to Aruga et al., the disclosure of each of which is incorporated herein by reference in its entirety.

Dosages. For all of the above molecules, as further studies are conducted, information will emerge regarding appropriate dosage levels for treatment of various conditions in various patients, and the ordinary skilled worker, considering the therapeutic context, age and general health of the recipient, will be able to ascertain the proper dosage. Generally, for injection or infusion, dosage will be between 0.01 μg of biologically active protein/kg body weight, (calculating the mass of the protein or complex alone, without chemical modification), and 10 mg/kg (based on the same). The dosing schedule may vary, depending on the circulation half-life of the protein(s) or derivative(s) used, whether they are delivered by bolus dose or continuous infusion, and the formulation used.

One method of treating a human having a provisional diagnosis of physiological obesity of the present invention includes administering doses of leptin bound to the leptin binding protein at a dose of 5 mg leptin/kg body weight intravenously, once a day for 30 days. The subject's blood levels of leptin bound to the leptin binding protein are serially monitored to determine if the administered dose is appropriate as indicated by normalization of the bound leptin level. The dose is adjusted in 1 mg leptin/kg body weight up or down as appropriate depending on the measured ratios until the level of bound leptin normalizes, and that dose is then maintained throughout the treatment period.

Administration with Other Compounds

For therapy associated with obesity, one may administer the leptin binding protein alone or in conjunction with leptin, including a complex thereof, (or derivatives) in conjunction with one or more pharmaceutical compositions used for treating other clinical complications of obesity, such as those used for treatment of diabetes (*e.g.*, insulin), high blood pressure, high cholesterol, and other adverse conditions incident to obesity. Also, other appetite suppressants may be co-administered, *e.g.*, amphetamines. Administration may be simultaneous (for example, administration of a mixture of the present protein and insulin) or may be *in seriatim*. Additionally,

when the treatment includes both the leptin binding protein and leptin the administration also may be simultaneous or *in seriatim*.

Nucleic Acid-based Therapeutic Treatment

The *leptin binding protein* gene could be introduced into human fat cells to develop gene therapy for obesity. Such therapy would be expected to decrease body weight. Conversely, introduction of antisense constructs into human fat cells would reduce the levels of active leptin binding protein and would be predicted to increase body adiposity.

In one embodiment, a gene encoding the leptin binding protein is introduced *in vivo* in a viral vector. Such vectors include an attenuated or defective DNA virus, such as but not limited to herpes simplex virus (HSV), papillomavirus, Epstein Barr virus (EBV), adenovirus, adeno-associated virus (AAV), and the like. Defective viruses, which entirely or almost entirely lack viral genes, are preferred. Defective virus is not infective after introduction into a cell. Use of defective viral vectors allows for administration to cells in a specific, localized area, without concern that the vector can infect other cells. Thus, adipose tissue can be specifically targeted. Examples of particular vectors include, but are not limited to, a defective herpes virus 1 (HSV1) vector [Kaplitt *et al.*, *Molec. Cell. Neurosci.*, **2**:320-330 (1991)], an attenuated adenovirus vector, such as the vector described by Stratford-Perricaudet *et al.*, *J. Clin. Invest.*, **90**:626-630 (1992), and a defective adeno-associated virus vector [Samulski *et al.*, *J. Virol.*, **61**:3096-3101 (1987); Samulski *et al.*, *J. Virol.*, **63**:3822-3828 (1989)].

In another embodiment, the gene can be introduced in a retroviral vector, *e.g.*, as described in Anderson *et al.*, U.S. Patent No. 5,399,346; Mann *et al.*, *Cell*, **33**:153 (1983); Temin *et al.*, U.S. Patent No. 4,650,764; Temin *et al.*, U.S. Patent No. 4,980,289; Markowitz *et al.*, *J. Virol.*, **62**:1120 (1988); Temin *et al.*, U.S. Patent No. 5,124,263; International Patent Publication No. WO 95/07358, published March 16, 1995, by Dougherty *et al.*; and Kuo *et al.*, *Blood*, **82**:845 (1993).

Alternatively, the vector can be introduced *in vivo* by lipofection. For the past decade, there has been increasing use of liposomes for encapsulation and transfection of nucleic acids *in vitro*. Synthetic cationic lipids designed to limit the difficulties and dangers encountered with liposome mediated transfection can be used to prepare liposomes for *in vivo* transfection of a gene encoding a marker [Felgner et al., *Proc. Natl. Acad. Sci. USA*, **84**:7413-7417 (1987); *see* Mackey *et al.*, *Proc. Natl. Acad. Sci. USA*, **85**:8027-8031 (1988)]. The use of cationic lipids may promote encapsulation of negatively charged nucleic acids, and also promote fusion with negatively charged cell membranes [Felgner *et al.*, *Science*, **337**:387-388 (1989)]. The use of lipofection to introduce exogenous genes into specific organs *in vivo* has certain practical advantages. Molecular targeting of liposomes to specific cells represents one area of benefit. It is clear that directing transfection to particular cell types would be particularly advantageous in a tissue with cellular heterogeneity, such as the pancreas, liver, kidney, and brain. Lipids may be chemically coupled to other molecules for the purpose of targeting (*see* Mackey *et al., 1988, supra*). Targeted peptides, *e.g.*, hormones or neurotransmitters, and proteins such as antibodies, or non-peptide molecules could be coupled to liposomes chemically.

It is also possible to introduce the vector *in vivo* as a naked DNA plasmid. Naked DNA vectors for gene therapy can be introduced into the desired host cells by methods known in the art, *e.g.*, transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun, or use of a DNA vector transporter (see, *e.g.*, Wu *et al.*, *J. Biol. Chem.*, **267**:963-967 (1992); Wu *et al.*, *J. Biol. Chem.*, **263**:14621-14624 (1988); Hartmut *et al.*, Canadian Patent Application No. 2,012,311, filed March 15, 1990).

Agricultural Applications

The *leptin binding protein* gene can also be isolated from domestic animals, and the corresponding protein obtained thereby. As discussed for human therapies, recombinant proteins can also be prepared and administered to domestic animals.

Administration of the polypeptide can be implemented to produce leaner food animals, such as beef cattle, swine, poultry, sheep, etc. Preferably, an autologous leptin binding protein is administered, although the invention contemplates administration of anti-autologous polypeptide as well.

Alternatively, the introduction of the cloned genes into transgenic domestic animals would allow one to potentially decrease body weight and adiposity by over-expressing a leptin binding protein transgene. The simplest means of achieving this would be to target a *leptin binding protein* transgene to liver or another factor-producing organ using its own or another organ specific promoter.

Conversely, increases in body fat might be desirable in other circumstances such as for the development of Kobe beef or fatty liver to make foie gras. This could be accomplished by targeting an antisense *leptin binding protein* transgene to fat, or by using gene knockout technology. Alternatively, where an increase in body weight at percentage of fat is desired, an inhibitor or antagonist of the leptin binding protein or (L-LBP) complex can be administered. Such inhibitors or antagonists include, but are not limited to, antibodies reactive with the protein and/or complex.

Cosmetic Implications

The leptin binding protein has significant value for cosmetic use, in addition to the health benefits. In particular, since the leptin binding protein and the (L-LBP) complex including derivatives and agonist analogs thereof, are useful for modulation of the rate and quantity of fat cell deposition in an animal, they are useful for reducing unsightly fat tissue, *e.g.*, fat deposits in the abdomen, hips, thighs, neck, and chin that do not necessarily amount to an obese condition, but which nevertheless detract from an individual's appearance. The fat reduction effect is thought to be accomplished, in part, by a reduction in appetite, *i.e.*, a reduction in food intake, by an increase in basal metabolism, or both. Thus, the leptin binding protein, or its derivatives or agonist

analogs, is useful for administration to a subject to effect cosmetic changes in fat tissue deposits, whether by modulating fat deposition, reducing appetite, or both.

In addition, the present compositions and methods may be used in conjunction with various procedures, such as cosmetic surgeries designed to alter the overall appearance of a body (*e.g.*, liposuction or laser surgeries designed to reduce body mass by aspirating or ablating fat tissue), exercise (especially running and weight training), low fat diet, hypnosis, biofeedback, as examples of the ways one may attempt to decrease the percentage of fat tissue and improve the appearance of the body.

Accordingly, the present invention relates to a method for effecting cosmetic fat tissue modulation in an individual comprising administering a fat modulating amount of leptin binding protein or the (L-LBP) complex or derivative or agonist analog thereof, to an individual who desires cosmetic fat tissue modulation to improve overall body appearance. In a particular aspect, the fat tissue modulation is a consequence of appetite suppression. Preferably, the fat tissue modulation is a reduction in fat tissue.

In a further embodiment, the invention relates to a method for effecting cosmetic fat tissue loss comprising combining a procedure for changing body appearance with administration of a fat modulating amount of the leptin binding protein or the (L-LBP) complex, or derivative or agonist analog thereof, to an individual who desires cosmetic fat tissue modulation to improve overall body appearance.

The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLES

EXAMPLE 1

Description of the purification and identification of leptin binding substance.

The association between leptin and the leptin binding protein (in particular apolipoprotein J) in the blood of humans and other mammals is demonstrated as summarized below and then described in greater detail as follows:

1) Polyclonal anti-leptin antibodies were produced to recombinant human leptin, as well as to recombinant mouse leptin.

2) Leptin affinity columns were prepared.

3) The anti-leptin antibodies were purified using the leptin affinity columns.

4) Anti-leptin affinity columns were prepared using the purified anti-leptin antibodies.

5) The anti-leptin affinity columns were used to separate material containing leptin from human blood samples.

6) The material containing human leptin that was separated using the anti-leptin affinity columns was analyzed using gel filtration to separate the components by relative molecular weight, and fractions of the eluate were analyzed to determine their immunoreactivity to anti-leptin antibodies. It appeared that leptin in human blood is associated, at least in part, with at least one protein that was labeled "leptin binding substance."

7) The processes used to analyze leptin from human blood samples were repeated using mouse blood samples to determine whether mouse leptin was also associated with a corresponding binding substance in mouse serum. It appeared that mouse leptin was associated with a corresponding binding substance.

8) Leptin affinity columns were used to affinity isolate the human leptin binding substance from human serum samples, and its molecular weight was determined by SDS-PAGE.

9) Rabbit anti-human leptin binding substance antibodies were produced, affinity purified and cross-absorbed against a panel of normal human proteins.

10) Biotinylation of the rabbit anti-human leptin binding substance antibodies and Horse radish peroxidase (HRP) conjugation of streptavidin was performed.

11) Human leptin binding substance was purified using the biotinylated rabbit anti-human leptin binding substance antibodies in conjunction with SDS-PAGE and western blotting.

12) The purified human leptin binding substance was sequenced and the identity of the leptin binding substance was confirmed to be apolipoprotein J.

Expanded description of the purification and identification of leptin binding substance.

1) *Production of Polyclonal Anti-leptin Antibodies*

Polyclonal antibodies to recombinant human leptin, as well as to recombinant mouse leptin were produced using New Zealand White rabbits. One group of animals received priming injections consisting of 1-2 mg of recombinant human leptin emulsified in Freund's complete adjuvant and subsequent booster injections containing 0.25-0.50 mg of recombinant human leptin emulsified in Freund's incomplete adjuvant. Another group of animals received identical injections containing recombinant mouse leptin. Booster injections were made at the fourth and fifth week following the primary injection. On the sixth week the rabbits were bled. The animals were rested till the eighth week and then placed on a continuous injection-bleed-rest cycle that began with a booster injection in the first week,

bleeding in the second week, and a 3-4 week rest period. The animals were maintained on the continuous injection-bleed-rest cycle, of approximately five- six weeks in length. Between 25-50 ml of blood was collected from the proximal ear vein of each animal at each bleed. This blood was allowed to clot overnight at 4°C, and then centrifuged for 30 min at 2000 g. The clarified serum was collected by aspiration and preserved by adding sodium azide at a final concentration of 0.1%.

2) *Preparation of Leptin Affinity Columns*

Leptin affinity columns were prepared as follows. SEPHAROSE 4B (available from Pharmacia Biotech, Piscataway, NJ) was washed thoroughly in water by repeated filling, mixing, settling and decanting. After washing, the SEPHAROSE 4B was suspended as a 50% slurry in 0.1 M carbonate buffer (pH 11). Cyanogen bromide (Sigma, St. Louis, MO) was dissolved to saturation at room temperature in DMF (Aldrich, Milwaukee, WI) and added to the SEPHAROSE slurry in a 1:10 v/v ratio. The activation procedure was carried out on ice, and in a fume hood with constant pH monitoring. The pH was maintained at 11 by adding 10 N NaOH as necessary. The activated material was washed in a vacuum funnel, first with chilled water, and then with a chilled borate buffer. Recombinant human leptin was added at a maximum of 10 mg of protein per ml of packed volume of SEPHAROSE. This material was allowed to stand for at least six hours at 4°C to permit the conjugation reaction to go to completion. Prior to use, the conjugated Sepharose was blocked with 1 M glycine and loaded into a column that was suitable for affinity purifying or cross-absorbing serum antibodies. Recombinant mouse leptin columns were prepared in a corresponding manner.

3) *Affinity Purification of Polyclonal Anti-leptin Antibodies*

Anti-leptin antibodies were purified using the leptin affinity columns as follows. One liter of hyperimmune rabbit antiserum was diluted to two liters with 0.34 M borate buffered saline pH 8.2 and passed over a leptin affinity column prepared as described above. The bound antibody was eluted using 0.1 M glycine-HCl (pH 3.0), followed

by 0.1 M glycine-HCl (pH 2.0), and followed by 0.1 N HCl containing 0.1 M NaCl. The eluted antibody was neutralized by adding excess borate buffered saline. F(ab')2 fragments were generated from the eluted antibody by dialyzing the antibodies into 0.1 M acetate buffer (pH 4.5), adding pepsin at a ratio of 2-3 mg per 100 mg of antibody, and incubating the mixture for between 4-18 hours at temperatures ranging between room temperature and 37°C. After incubation, the mixture was neutralized and purified by passage through a column of immobilized protein G, and then by passage through a column of SEPHADEX G-200.

4) *Preparation of Anti-leptin Affinity Columns*

Anti-leptin affinity columns were prepared using the purified anti-leptin antibodies in a manner corresponding to the preparation of leptin affinity columns, described above.

5) *Purification of Leptin Containing Material from Human Blood*

Leptin containing material was separated from human blood samples using the anti-leptin affinity columns. Human blood samples were obtained from patients undergoing a phlebotomy unrelated specifically to obesity. The samples were initially collected using a variety of procedures, including with and without heparin, sodium citrate, and EDTA. Soluble serum and plasma proteins in the samples were separated from cellular material by centrifugation at 1000-2000 g for 30-60 minutes. The soluble fraction was decanted and the cellular material was discarded. The serum was pooled and passed over the anti-leptin affinity columns containing immobilized rabbit anti-leptin antibody prepared as described above. The retained material was eluted using 0.1 M glycine-HCl (pH 3.0), followed by 0.1 M glycine-HCl (pH 2.0), and followed by 0.1 N HCl containing 0.1 M NaCl. The eluted material (containing leptin) was neutralized by the addition of an excess of borate buffered saline and was concentrated by ultrafiltration using a pressurized stirred cell concentrator equipped with a 10 kD cutoff membrane (Amicon, Beverly, MA).

6) *Analysis of Material Containing Human Leptin*

The material containing human leptin that was separated using the anti-leptin affinity columns was divided into analytical and preparative portions and analyzed by gel filtration through SUPEROSE 6 and SUPEROSE 12 (Pharmacia, Piscataway, NJ). The SUPEROSE was packed into preparative glass FPLC columns which were hooked together in series, and pumped at 1 ml/min using standard HPLC equipment (SSI, State College, PA). The columns used to analyze the analytical portion were equilibrated with phosphate buffered saline (pH 7.2). Some columns used to analyze the preparative portion were equilibrated with neutral pH, non-dissociating buffer (0.34 M Borate buffered saline) similar to the analytical portion, while other of the columns used to analyze the preparative portion were equilibrated with acidic pH, mild, dissociating buffer (0.1 M HCl containing 0.1 M NaCl). The resultant fractions produced by the gel filtrations were collected in 1 ml aliquots. The preparative fractions were further evaluated for immunoreactive material by direct ELISA. The ELISA was performed by adding of 100 μl of 0.34 M borate buffer pH 8.2 to each well of a 96 well plate. Aliquots of 100 μl of each fraction was added to sequential wells on the plate and the solutions in the individual wells were thoroughly mixed. Serial dilutions were performed by removing 100 μl from each well, adding it to the next successive well, mixing and then repeating the process. This results in dilutions of 1:2, 1:4, 1:8 and so on of the original aliquots. The material was allowed to bind directly and passively to the high binding flexible ELISA plates (purchased from Costar) by overnight incubation at 4° C.

The next day the plates were washed of excess unbound antibodies and blocked with 0.34 M borate buffered saline containing 0.1% tween-20, 0.1% gelatin, and 1% BSA, and then probed with biotinylated rabbit anti-leptin. The plates were then washed again, and reprobed with HRP (horse radish peroxidase) conjugated streptavidin. The plates were washed once more and then developed with ABTS (2,2'-Azinobis(3-ethylbenzthiazolinesulfonic acid)) substrate and read on a plate reader.

Figure 1 shows a continuous trace, gel filtration profile under neutral pH and monitored at 280 nm, showing the molecular weight distribution of the proteins in the analytical run. As can be seen, the material eluted in fractions represented by a large high molecular weight peak, a second large, intermediate weight peak, and a small, low molecular weight peak. Based on the known weight of recombinant leptin, if free leptin were present in the preparation it would have eluted in fractions corresponding to the low molecular weight peak. This analysis indicated that the human blood-borne leptin which eluted from the column was partly free, and partly bound directly to two carriers, or alternately partly free, partly bound or associated with two high molecular weight complexes. As will be developed below, these complexes probably correspond to a complex of leptin and leptin binding protein, and a complex of the former in a lipoprotein particle.

Figure 2 shows a plot of immunoreactivity versus elution volume (fraction number) of fractions collected from the eluate of the gel filtration columns under non-dissociating conditions (neutral pH). The plot shows how immunoreactive leptin was partitioned along with the proteins which were separated by size by the gel filtration column under non-dissociating conditions (neutral pH). The level of immunoreactive leptin in each fraction was measured by direct binding ELISA probed with a biotinylated affinity purified polyclonal rabbit anti-human leptin antibodies. As can be seen, immunoreactive leptin eluted in high molecular weight fractions, inconsistent with the known leptin molecular weight. Based on the known molecular weight of recombinant leptin, free leptin would have eluted from the column throughout fractions 32-34. The apparent lack of free leptin in these fractions suggested that variable proportions of leptin in normal, non-obese, humans is associated with other substances rather than free.

Referring now to Figure 3, there is shown a semi-continuous plot of immunoreactivity versus elution volume (fraction number) of fractions collected from the eluate of the gel filtration columns under dissociating conditions (acidic pH). The plot shows how

immunoreactive leptin was partitioned along with the proteins which were separated by size by the gel filtration column under dissociating conditions (acidic pH). The level of immunoreactive leptin in each fraction was measured using a polyclonal rabbit anti-human leptin antibody-based immunoassay (closed squares) and a mono-specific, polyclonal antibody-based assay (closed diamonds). The mono-specific, polyclonal antibody was raised against a synthetic peptide having an amino acid sequence of VPIQKVQDDTKTLIKTIVT, which is unique to leptin but common to both mouse and human leptin. Rabbits were immunized with recombinant protein in Freund's adjuvant (HRP, Inc.).

As can be seen, under dissociating conditions, there was a decrease in the level of immunoreactive leptin in the higher molecular weight fractions compared with non-dissociating conditions (especially in the intermediate weight peak shown in Figure 1). Under these conditions, immunoreactive leptin appeared in fractions 33-37, which corresponds to the known molecular weight of recombinant leptin. The mono-specific, polyclonal antibody (closed diamonds) was virtually unable to detect leptin in the high molecular weight fractions or to detect fragments which migrated to positions of lower molecular weight than intact leptin. Therefore, the mono-specific, polyclonal anti-recombinant leptin antibodies appeared to have specificity for a region of leptin that was sensitive to degradation when leptin is fragmented, and that was also involved in binding with a substance of higher molecular weight or close enough to a region responsible for binding a substance to cause steric hindrance with respect to the binding of the anti-leptin antibodies.

These data indicate that leptin in the human blood samples was, at least in part associated with one or more substances having a higher molecular weight than free leptin in normal human serum. The analyses also implied that the association between leptin and the substance was at least in part noncovalent since some free leptin dissociated from the complex under mild dissociating conditions. This material that was apparently binding leptin was initially labeled "leptin binding substance."

7) *Analysis of Mouse Serum Leptin and Comparison to Human Serum Leptin*

In order to determine whether mouse leptin was also associated with a binding substance in normal mouse serum, the processes described above used to analyze leptin from human blood samples were repeated using rabbit anti-mouse-leptin antibodies, and commercially available normal mouse serum (Bioproducts, Indianapolis, IN). The results were compared with corresponding analyzes for human blood samples. The eluted material was analyzed in a manner corresponding to that which produced Figure 1 and the resulting profile (not shown) was substantially identical to that shown in Figure 1. Further, the eluted material was fractionated and tested in a manner corresponding to that which produced Figure 2 and the resulting graph (not shown) was also substantially identical. The material was further fractionated and tested in a manner corresponding to that which produced Figure 3 and these results are shown in Figure 4, which is a semi-continuous plot of immunoreactivity versus elution volume (fraction number) of fractions collected from the eluate of the gel filtration columns under dissociating conditions (acidic pH) for mouse blood (open diamonds). A profile of human serum derived leptin containing material is overlaid for comparison.

As can be seen in Figure 4, under dissociating conditions, immunoreactive low molecular weight (unbound) mouse serum leptin eluted from the column in the same numbered fractions (36-41) (closed diamonds) as did immunoreactive human serum leptin (open squares). One difference observed was that the mono-specific polyclonal antibody used as the probe in this assay for immunoreactive leptin detected mouse leptin in the high molecular weight fractions which implied a difference in the physical interaction between mouse leptin and the mouse leptin binding substance in the mouse serum compared with the interaction between human leptin and the human leptin binding substance (results discussed above).

8) *Initial Isolation, Purification and Molecular Weight Characterization of Leptin Binding Substance*

In order to characterize the leptin binding substance, it was purified from human serum. Human blood samples were obtained from multiple donors undergoing a phlebotomy for disorders unrelated to obesity. Soluble serum and plasma proteins were separated from cellular material by centrifugation at 1000-2000 g for 30-60 min. The soluble fraction was decanted off and the cellular material was discarded. The serum from the multiple donors was pooled and passed over leptin-affinity columns containing immobilized recombinant human leptin. Bound material was eluted using a 0.1% solution of SDS prewarmed to 37°C in order to assure complete dissociation of noncovalent complexes formed between the immobilized recombinant leptin and the serum derived leptin binding substance. The eluted material was concentrated and excess SDS was removed by repeated diafiltration in a borate buffered saline also prewarmed to 37°C using stirred cell equipment and a 10 kD pore size ultrafilter (Amicon, Beverly, MA). The eluate was then subjected to FPLC analysis and purification in a manner corresponding to the FPLC analysis of blood-derived leptin, described above.

The purified material was analyzed by SDS-PAGE on 5-15% gradient gels (Biorad, Richmond, CA). The results are shown in Figure 5. The material was analyzed under both non-reducing (A) and reducing conditions (B). The gels were stained with Coomassie Blue, destained, and dried all according to standard methods. As can be seen, there was a predominant band in the region corresponding to a molecular weight of 80 kD under non-reducing conditions and a molecular weight of 40 kD under reducing conditions, lanes 1 and 3. Lanes 2 and 4 represent molecular weight markers.

9) *Affinity Purification and Cross Absorption of Rabbit Anti-human Leptin Binding Protein Antibodies*

Polyclonal rabbit, anti-human leptin binding substance antibodies were produced to the material present in the 40 kD band, isolated as described above, in a manner corresponding to the production of polyclonal antibodies to leptin, also described above. Leptin binding substance affinity columns were produced in a manner corresponding to the production of leptin affinity columns, described above, using the same material present in the 40 kD bands, described above, which was bound to cyanogen bromide activated SEPHAROSE. The rabbit anti-human leptin binding substance antibodies were affinity purified on the leptin binding substance columns, in a manner corresponding to that described above for the antibodies to leptin.

In addition to affinity purification, the rabbit anti-human leptin binding substance antibodies were cross-absorbed against a large panel of purified human proteins including immunoglobulins, collagens, and albumin. The proteins were extracted from a variety of human tissues, including serum, in an attempt to assure that naturally occurring and potentially problematic antibodies in the rabbit serum were discarded from the final preparation and that antibodies raised unintentionally in the rabbits in response to the minor contaminants, which might be present in the isolated leptin binding substance, were also removed and discarded. The level of cross-reactive antibody depletion was monitored by calculating the amount of protein lost following each pass and by monitoring the amount of protein which bound to the columns during the subsequent washing and recycling. As expected, only a small fraction of the total antibody bound to the affinity column and most of the antibody which was lost during the cross-absorption was lost during the first absorptions indicating nonspecific cross reactions.

10) *Biotinylation of Anti-leptin Binding Substance Antibody and HRP Conjugation of Streptavidin*

Long chain N-hydroxysuccinimidobiotin (NHS biotin) (Sigma, St. Louise, MO), was dissolved in N,N-dimethylformamide (DMF) (Aldrich, Milwaukee, WI) and added to the anti-human leptin binding substance antibody at a molar ratio of approximately 15:1. The mixture was incubated for 30-60 minutes and free biotin was removed by gel filtration on SEPHADEX G-25. The conjugated material was diluted into 1% bovine serum albumin (BSA) at a final concentration of 1 mg/ml. Streptavidin at a concentration of 10 mg/ml was dialyzed into 0.1 M carbonate buffer, pH 9.5. HRP having a concentration of 10 mg/ml was dialyzed into 0.1 M carbonate buffer at pH 8.0. Sodium periodate was added to the HRP at approximately 1 μg/ml. After 2-4 hours at room temperature, the periodate was inactivated by adding of 1/6 w/v or dry G-25 and the dialyzed streptavidin was added to the mixture. The HRP/streptavidin mixture was allowed to stand for between 18 and 24 hours. The conjugate was then stabilized with sodium borohydride having a final concentration of 1 μg/ml, purified by gel-filtration on Superose 6 in order to exclude over and under conjugated material, and diluted into 1% BSA at a final concentration of 1 mg/ml.

11) *SDS-PAGE and Western Blotting of Human Serum with Anti-Leptin Binding Substance*

The specificity of the antibody raised to the leptin binding substance was first analyzed by Western blotting of normal human serum as follows. One hundred μl of normal human serum was run on 5-15% SDS-PAGE as described above under both reducing and non-reducing conditions using standard methods and equipment (Biorad, Richmond, CA). The resulting slab gels were removed from the apparatus and the separated proteins were transferred to nitrocellulose blotting membranes (Costar, Cambridge, MA) using a standard submersion tank electrophoretic transfer apparatus (Biorad, Richmond, CA). Each gel was blotted twice. The blotted nitrocellulose membranes were lifted from the slab gel and transferred to 0.34 M borate buffered saline containing 0.1% gelatin and 1% BSA, and 0.1% tween-20 (BMB, Indianapolis,

IN). The slab gel was transferred to a solution of Coomassie Blue. The blots were incubated for 2 hours, washed several times, and the primary antibody was added, all in the same buffer. Unprocessed antiserum was added to one set of reduced and non-reduced blots at a final dilution of 1:1000. The affinity purified, cross-absorbed, and biotinylated anti-human leptin binding substance antibodies were added to the other set of blots at a final dilution of 1:10,000 from the original 1 mg/ml stock. After incubation overnight, both sets of blots were washed again. The first set of blots was counter-stained with biotinylated goat anti-rabbit IgG also diluted 1:10,000 (human serum absorbed from Brookwood Biomedical, Birmingham, AL, also available from Sigma, St. Louis, MO) for 2 hours. Both sets of blots were again washed, incubated with a 1:1,000 dilution of alkaline phosphatase conjugated streptividin (Brookwood Biomedical, Birmingham, AL, also available from Sigma, St. Louis, MO) for an additional 2 hours, and washed again. Finally, the blots were transferred to 100 ml of substrate buffer consisting of 0.1 M carbonate buffer pH 9.5 containing 15 mg of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 30 mg of Nitro Blue Tetrazolium (NBT) (Molecular Probes, Eugene, OR). Color was allowed to develop and was monitored visually. The blots were then air dried.

Referring now to Figure 6, there is shown a Coomassie Blue stained, 5-15% SDS-PAGE analysis of the whole, unfractionated normal human serum from nine different normal, non-obese individuals under non-reducing conditions (A) and reducing conditions (B). The rightmost lane (#10) contains molecular weight standards. As can be seen, each lane containing human serum presents a pattern similar to the other lanes and displays a significant albumin band and significant immunoglobulin bands. In addition, there are present a large number of less prominent, high and low molecular weight bands corresponding to various normal human serum proteins.

Referring now to Figure 7, there is shown a nitrocellulose immunoblot of an SDS-PAGE gel as shown in Figure 6, using unfractionated and unprocessed rabbit

anti-leptin binding substance anti-serum generated as above, under both non-reducing (A) and reducing (B) conditions. As can be seen, the antiserum displayed significant cross-reactivity with a variety of serum proteins on both the reduced and non-reduced immunoblots.

Referring now to Figure 8, there is shown a nitrocellulose immunoblot of an SDS-PAGE gel as shown in Figure 6, using the affinity purified and cross-absorbed rabbit anti-human leptin binding substance antibodies prepared as above, under non-reducing conditions (A) and reducing conditions (B). As can be seen, the antibody displayed specificity for a component of normal human serum with an apparent molecular weight of approximately 80 kD and another component with an apparent molecular weight of approximately 40 kD under non-reducing conditions (A). Under reducing conditions (B), the antibody showed specificity for one component with an apparent molecular weight of approximately 40 kD. This suggests that leptin binding substance can exist either as a monomer or as a dimer crosslinked by disulfide bonds.

12) *Confirming the Identity of Leptin Binding Substance though Amino Acid Sequencing*

In order to confirm the identity of leptin binding substance, aliquots of leptin affinity column eluate were concentrated, dialyzed against saline, and further processed by gel filtration on Superose 12. These steps were taken both in order to remove excess SDS for better resolution on SDS-PAGE and to remove contaminants which could potentially make it difficult to remove the 40 kD band for extraction and N-terminal amino acid sequencing. The material eluted as a somewhat broad band from the Superose 12 column and four fractions were collected throughout the elution which was monitored at 280 nm. These fractions were analyzed by SDS-PAGE and the cleanest fraction was chosen for sequencing.

Referring now to Figure 9, there is shown a Coomassie Blue stained 5-15% SDS-PAGE of each of the four fractions run under reducing conditions in individual lanes. The gel shows four individual lanes which correspond to the four fractions collected sequentially from the leptin affinity gel-filtration column. Lane 1 (leftmost) corresponded to the fraction with the highest molecular weight, lane 4 (from the left) corresponded to the fraction with the lowest molecular weight and lanes 2 and 3 corresponded to the fractions of intermediate weight. As can be seen, lanes 1 and 2 show a predominant band in the molecular weight region of 40 kD, the weight corresponding to the leptin binding substance, and lanes 3 and 4 show predominantly low molecular weight contaminating materials. Based on this visual assessment, the material shown in lane 1 which corresponds to the material in fraction 1 was sent for N-terminal amino-acid sequencing according to techniques well known to those with skill in the art. The sequencing revealed that leptin binding substance was apolipoprotein J, a substance previously known to have a variety of functions, but not previously known to have an association with leptin. The results of the analyses above are consistent with known characteristics of apolipoprotein J since apolipoprotein J is known to exist as a disulfide cross-linked homodimer with a monomeric size of 40 kD and a dimeric size of 80 kD. The apolipoprotein J precursor is linked *via* a disulfide bond and post-translationally cleaved. The resulting 80 kD molecule consists of a heterodimer between an apolipoprotein J α and an apolipoprotein β chain, each of which is about 40 kD. The α and β chains joined in this heterodimer are derived from the same precursor. Apolipoprotein J is also known to associate with high molecular weight lipoprotein complexes (especially HDL). These characteristics of apolipoprotein J contributed to the confirmation that in at least one embodiment leptin binding substance is apolipoprotein J.

EXAMPLE 2

Assay of bound and unbound leptin

According to another aspect of the present invention, there is provided an immunoassay of bound and unbound leptin. The assay used

2,2'Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as the substrate, and used two different signaling antibodies, one specific for human leptin and the other specific for protein binding protein. Both antibodies were conjugated to biotin and used in conjunction with HRP-streptavidin, prepared using techniques described above. As will be understood by those having skill in the art with reference to the disclosure herein, other detection methods can be used including I-125, fluorescein isothiocyanate (FITC), phycoerythrin, alkaline phosphatase, horseradish peroxidase, and B-galactosidase, as well as other substrates such as 3,3,'5,5' tetramethylbenzidine (TMB) and o-phenylene diamine (OPD). Similarly, with reference to the disclosure herein such skilled artisans would know of the varied means of using and monitoring these methods. Two sets of two flexible high protein binding 96 well ELISA plates (Costar, Cambridge, MA) were passively coated with polyclonal rabbit, anti-human leptin antibodies prepared as described above.

The rabbit anti-human leptin antibody was passively coated directly onto ELISA plates by adding 100 μl of a 10 μg/ml solution of the antibody diluted in 0.34 M borate buffered saline (pH 8.2) for 18-24 hrs. at 4°C. The plates were washed of excess unbound antibodies and blocked with 0.34 M borate buffered saline containing 0.1% tween-20, 0.1% gelatin, and 1% BSA. Twenty-four human serum samples were obtained, which included samples from seven patients with anorexia nervosa (#1-7), samples from five normal donors (#8-12), samples from eight obese Pima Indians (#13-20), and samples from four patients with Prader-Willi Syndrome (#21-24) (a congenital disease that causes obesity in the affected individuals). One hundred μl of each serum sample was added at dilutions of 1:2 and 1:20 in the above buffer to individual wells of the assay plate and allowed to incubate for 24 hrs at 4°C. The plates were again washed. One hundred μl of a 1:20,000 dilution (from a 1 mg/ml stock) of biotinylated anti-human leptin antibodies were added to one set of plates, while a similar dilution of biotinylated anti-human leptin binding protein antibodies (from a similar 1 mg/ml stock) was added to the other set of plates. The plates were allowed again to incubate for 2 hrs at room temperature. They were washed again and

100 μl of a 1:10,000 dilution of HRP-streptavidin was added and incubated for 2 additional hours. Finally, the plates were washed and ABTS substrate was added (BMB, Indianapolis, IN). After 10 minutes, the plates were read using an automatic plate reader (Titertec, ICN, Costa Mesa, CA).

Referring now to Figure 10, there is shown a scatter diagram summary of the results of these 2 parallel assays. The data are presented in terms of optical density (OD) units. Coordinates are given for anorexic (AN), normals (NO), obese (OB) and Prader-Willi (PW) subjects. The optical density measurements shown along the X axis were from the symmetrical assay based on the use of the anti-leptin antibodies to capture and biotin labeled anti-leptin antibodies to signal. This value appears to relate to the amount of leptin not associated with the leptin binding protein. The optical density measurements shown along the Y axis were from the asymmetrical assay based on the use of anti-leptin antibodies to capture and labeled anti-leptin binding protein antibodies to signal. These measurements represent a measure of the amount of leptin associated with leptin binding protein.

Figure 10A shows the data generated using the above ELISA formats and the human serum samples tested at a 1:20 dilution. It can be seen that two distinct distributions are apparent. One distribution (upper left) corresponded to data from normal and lean anorexic patients. The second distribution (lower right) corresponds to data from obese and Prader-Willi patients. As can be seen from the data, a substantial portion of serum leptin is associated with leptin binding protein in normal and lean anorexic individuals, but far less serum leptin in obese individuals is associated with leptin binding protein. Within this first distribution, the lean anorexic group is distinctly to the left of the normals, indicating that as a group an even higher percentage of their leptin is associated with the leptin binding protein.

Figure 10B shows data from human serum samples tested at a 1:2 dilution. Again, two distributions are apparent. One distribution (far left) corresponds to data from

normal and lean anorexic patients. A second distribution (middle to right) corresponds to data from obese and Prader-Willi patients. A substantial portion of serum leptin is associated with the leptin binding protein in normal and lean anorexic individuals since values along the Y axis remained higher than those along the X axis. Further, the low levels of unbound leptin in the serum of normal and lean anorexic individuals appear to compete more efficiently for binding to the anti-leptin antibody than bound leptin since the values along the Y axis are lower at the 1:2 dilution than at the 1:20 dilution.

The data indicate that the symmetrical assay used herein may be affected by steric hindrance when a substantial portion of leptin is bound to the leptin binding protein. Such steric hindrance probably accounts for discrepancies between the serum leptin levels obtained in the symmetrical assay herein and results derived using other immunoassay formats including both immunoprecipitation and semi-quantitative immunoblotting, and single site competitive radioimmunoassays. Hence, standard assays for leptin levels may have greater sensitivity for leptin levels in obese individuals (because leptin is largely unbound to the leptin binding protein in such individuals) than leptin levels in normal or lean individuals.

The asymmetrical assay format used herein appears to provide a more accurate measure of total serum leptin levels in normal and lean anorexic individuals than standard assays for leptin, because the majority of the leptin appears to be associated with leptin binding protein in such individuals. In obese individuals, however, the asymmetrical assay does not appear to measure total serum leptin levels because the majority of the serum leptin in these individuals does not appear to be associated with leptin binding protein.

Therefore, previously used assays appear to have been underestimating the amount of total serum leptin in normal individuals due to the assays' lack of sensitivity for measuring leptin associated with the leptin binding protein. However, previously

used assays more accurately measured the amount of total serum leptin in obese individuals because only a small portion of leptin is associated with the leptin binding protein in such individuals.

Thus, it appears that the defect in the endogenous leptin pathway in obese individuals is not related solely to total leptin levels, but to the form of circulating leptin, whether bound or unbound to the leptin binding protein.

EXAMPLE 3:

The relationship of leptin to leptin bound to the leptin binding protein in AIDS patients.

As detailed above, there appears to be a correlation between the percentage of free leptin measured in a serum sample and obesity. Thus, the ratio of the total leptin concentration measured in a serum sample relative to the corresponding leptin bound to the leptin binding protein (L/L-LBP) increases with obesity and decreases with anorexia. To further probe the validity of this result serum samples from patients suffering for AIDS were examined since it is well documented that one of the symptoms of AIDS is an anorexic wasting.

The results of a study with AIDS patients in comparison with normal controls is shown in Figure 11. Figure 11A shows a plot of the percentage of body fat determined from serum samples of individual controls (x-axis) *versus* the measured L/L-LBP (y-axis). The relative amounts of total leptin and the leptin bound to the leptin binding protein were determined as described for Example 2.

As shown in Figure 11A, there is a reasonably linear relationship between the L/L-LBP in normal controls and their corresponding percentage of body fat. The serum of the AIDS patients also shows a linear correlation between the percentage of body fat of the subject and the amount of bound leptin (Figure 11B) but the slope of this plot is

significantly more shallow. Therefore, AIDS patients show a higher percentage of leptin complexed with the leptin binding protein at all levels of percent body fat. These results are consistent with the leptin-leptin binding protein complex playing a critical role in the leptin endogenous pathway.

The following is a list of documents related to the above disclosure and particularly to the experimental procedures and discussions. These documents, and all others cited above, should be considered as incorporated by reference in their entirety.

Bahary *et al.*, *Genomics*, **11**:33-47 (1991).

Bahary *et al.*, *Genomics*, **13**:761-769 (1992).

Bahary *et al.*, Molecular mapping of mouse chromosomes 4 and 6: Use of a flow-sorted Robertsonian chromosome (1991).

Blank *et al.*, *Mammalian Genome*, **1**:s51-s78 (1991).

Bogardus *et al.*, *Annals of the New York Academy of Sciences*, **630**:100-115 (1991).

de Silva *et al.*, *Biochemistry*, **29**: 5380-5389 (1990).

Friedman *et al.*, *Mammalian Genome*, **1**:130-144 (1991).

Harris, *FASEB J.*, **4**:3310-3318 (1990).

Jacobowitz *et al.*, *N. Engl. J. Med.*, **315**:96-100 (1986).

Kessey, in *Obesity*, pp. 144-166, Stunkard ed., Philadelphia, W.B. Sauders Co. (1980).

Kessey *et al.*, *Ann. Rev. Psychol.*, **37**:109-133.22 (1986).

Kirszbaum, L. *et al.*, *EMBO J.*, **8**: 711-718 (1989).

Jordan-Starck, T.C. *et al.*, *Journal of Lipid Research*, **35**: 194-210 (1994).

Leibel *et al.*, "Genetic variation and nutrition in obesity: Approaches to the molecular genetics of obesity", in *Genetic Variation and Nutrition*, pp. 90-101.1, Simopoulos and Childs eds., S. Karger, Basel (1990).

Siegel *et al.*, *Cytogenet. Cell Genet.*, **61**(3):184-185 (1992).

The present invention is not to be limited in scope by the specific embodiments describe herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description.

Various publications in addition to the immediately foregoing are cited herein, the disclosures of which are incorporated by reference in their entireties.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Lallone, Roger
Friedman, Jeffrey M.

(ii) TITLE OF INVENTION: LEPTIN BINDING PROTEIN AND ITS USE IN METHODS FOR DIAGNOSING AND TREATING ABNOMALITIES OF THE ENDOGENOUS LEPTIN PATHWAY

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
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(B) STREET: 411 Hackensack Ave, Continental Plaza, 4th Floor
(C) CITY: Hackensack
(D) STATE: New Jersey
(E) COUNTRY: USA
(F) ZIP: 07601

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/699,029
(B) FILING DATE: 16-AUG-1996
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Jackson Esq., David A.
(B) REGISTRATION NUMBER: 26,742
(C) REFERENCE/DOCKET NUMBER: 600-1-163

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 201-487-5800
(B) TELEFAX: 201-343-1684

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Xaa Gln Thr Val Ser Asp Asn Glu Leu Gln Glu Met Ser Asn Gln Gly
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Ser Lys Tyr Val Asn Lys Glu Ile
20

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Val Pro Ile Gln Lys Val Gln Asp Asp Thr Lys Thr Leu Ile Lys Thr
1 5 10 15

Ile Val Thr

WHAT IS CLAIMED IS:

1. A composition comprising a purified formulation of leptin and a leptin binding protein, wherein the leptin binding protein has the following characteristics:

(a) it co-purifies with leptin when leptin is purified on a leptin affinity column;

(b) it has a binding affinity for leptin; and

(c) it has an apparent molecular weight of about 80 kD determined by SDS-PAGE under non-reducing conditions and an apparent molecular weight of about 40 kD determined by SDS-PAGE under reducing conditions.

2. The composition of Claim 1 wherein the leptin binding protein has an N-terminal amino acid sequence of SEQ ID NO:1.

3. The composition of Claim 2 wherein the leptin binding protein is apolipoprotein J.

4. The composition of Claim 1 wherein the leptin binding protein is mammalian.

5. The composition of Claim 4 wherein the leptin binding protein is human.

6. The composition of Claim 5 wherein leptin is human.

7. An antibody specific for an epitope created by the association of leptin and the leptin binding protein of the composition of Claim 1.

8. A use of a composition comprising purified leptin binding protein and a pharmaceutically acceptable carrier for treating a mammalian subject suffering from an abnormality in the endogenous leptin pathway resulting in a decreased leptin activity wherein the leptin binding protein has the following characteristics:

(a) it co-purifies with leptin when leptin is purified on a leptin affinity column;

(b) it has a binding affinity for leptin; and

(c) it has an apparent molecular weight of about 80 kD determined by SDS-PAGE under non-reducing conditions and an apparent molecular weight of about 40 kD determined by SDS-PAGE under reducing conditions.

9. The use of the composition of Claim 8, wherein the composition further comprises leptin.

10. A method of detecting leptin bound to a leptin binding protein in a sample comprising the steps of:

(a) contacting a sample with a binding partner specific for a leptin-leptin binding protein complex, under conditions that allow the binding partner to associate with leptin-leptin binding protein complex present in the sample; and

(b) detecting binding of the binding partner to the leptin-leptin binding protein complex, wherein detection of binding of the binding partner to the leptin-leptin binding protein complex indicates the presence of leptin bound to the leptin binding protein in the sample; wherein the leptin binding protein has the following characteristics:

(i) it co-purifies with leptin when leptin is purified on a leptin affinity column;

(ii) it has a binding affinity for leptin; and

(iii) it has an apparent molecular weight of about 80 kD determined by SDS-PAGE under non-reducing conditions and an apparant molecular weight of about 40 kD determined by SDS-PAGE under reducing conditions.

11. A method of quantifying leptin bound to a leptin binding protein in a sample comprising detecting the presence of leptin bound to the leptin binding protein by the method of Claim 10, and further comprising

a) contacting a sample with a first binding partner, wherein said first binding partner binds to leptin under conditions that allow for binding;

b) contacting the sample with a second binding partner, wherein said second binding partner binds to the leptin binding protein under conditions that allow for binding; and

c) detecting binding of both the first binding partner and the second binding partner to a complex of leptin associated with leptin binding protein.

12. The method of Claim 10 wherein the binding partner for the leptin-leptin binding protein is selected from the group consisting of an anti-leptin antibody, an anti-leptin binding protein antibody, an antigen binding fragment thereof, a derivative thereof and a combination thereof.

13. A method for diagnosing an abnormality in the endogenous leptin pathway in a mammalian subject comprising the steps of:

(a) determining the amount of a form of apolipoprotein J in a biological sample acquired from the mammalian subject; and

(b) comparing the amount of said form of apolipoprotein J determined in the biological sample to a range of amounts of said form of apolipoprotein J determined in mammals having a normal endogenous leptin pathway and wherein the leptin binding protein has the following characteristics:

(i) it co-purifies with leptin when leptin is purified on a leptin affinity column;

(ii) it has a binding affinity for leptin; and

(iii) it has an apparant molecular weight of about 80 kD determined by SDS-PAGE under non-reducing conditions and an apparant molecular weight of about 40 kD determined by SDS-PAGE under reducing conditions.

14. The method of Claim 13 wherein diagnosing for the abnormality in the endogenous leptin pathway pertains specifically to diagnosing for physiological

obesity, wherein the form of apolipoprotein J is total apolipoprotein J; and wherein a determined amount from step (a) less than said range of amounts indicates a diagnosis of physiological obesity.

15. The method of Claim 13 wherein diagnosing for the abnormality in the endogenous leptin pathway pertains specifically to diagnosing for physiological obesity; wherein the form of apolipoprotein J is apolipoprotein J bound to leptin; and wherein a determined amount from step (a) less than said range of amounts indicates a diagnosis of physiological obesity.

16. A method for diagnosing an abnormality in the endogenous leptin pathway in a mammalian subject comprising the steps of:

(a) determining at least one parameter in a biological sample acquired from the mammalian subject selected from the group consisting of (i) an absolute amount of leptin bound to apolipoprotein J, (ii) an absolute amount of leptin not bound to apolipoprotein J, (iii) a percent of total leptin that is bound to apolipoprotein J, (iv) a percent of total leptin that is not bound to apolipoprotein J, (v) the ratio of (an absolute amount of leptin that is bound to apolipoprotein J) to (an absolute amount of total leptin), (vi) the ratio of (an absolute amount of leptin that is bound to apolipoprotein J) to (an absolute amount of leptin that is not bound to apolipoprotein J), and (vii) the ratio of (an absolute amount of leptin that is not bound to apolipoprotein J) to (an absolute amount of total leptin); and

(b) comparing the determined parameter with a range of values for the parameter in the same type of mammal without abnormalities in the endogenous leptin pathway

wherein a determined amount lesser or greater than the range of values indicates a diagnosis of an abnormality in the endogenous leptin pathway.

17. A method of monitoring the treating of an abnormality in the endogenous leptin pathway in a subject mammal by the administering of at least one dose of a

composition comprising leptin and apolipoprotein J, comprising the step of serially monitoring a value determined for a property of a biological sample acquired from the mammalian subject, the property being selected from the group consisting of

(i) the quantity of leptin bound to apolipoprotein J,

(ii) the quantity of leptin not bound to apolipoprotein J,

(iii) a quantitative relationship between (i) and (ii)

(iv) a quantitative relationship between the quantity of total leptin and (i) or (ii).

18. A kit for performing the method of Claim 11, comprising:

(a) a container holding the first binding partner; and

(b) a container holding the second binding partner.

19. The kit of Claim 18, further comprising a container holding purified leptin as a standard.

20. The kit of Claim 19, further comprising a container holding purified apolipoprotein J as a standard.

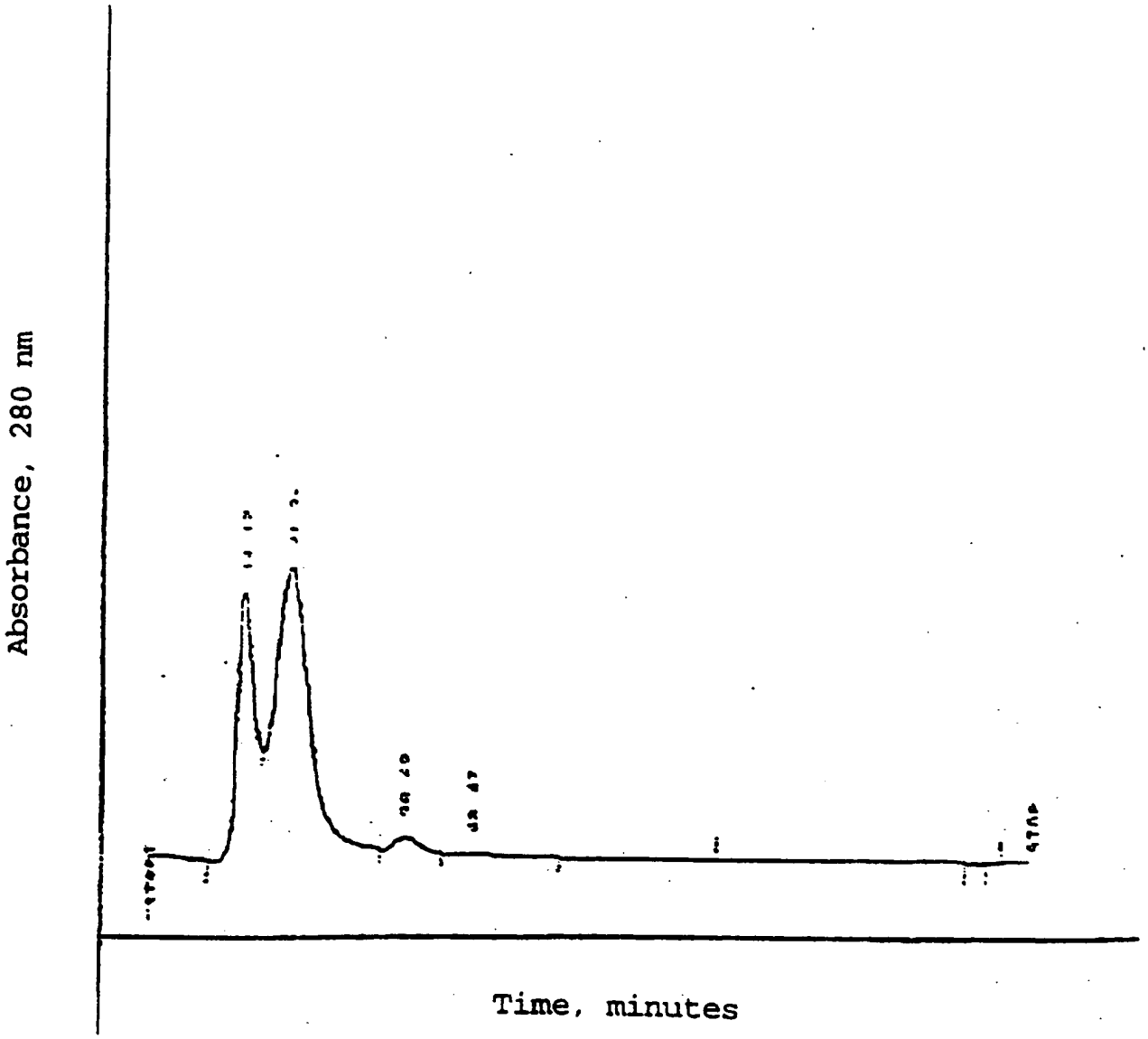

Figure 1

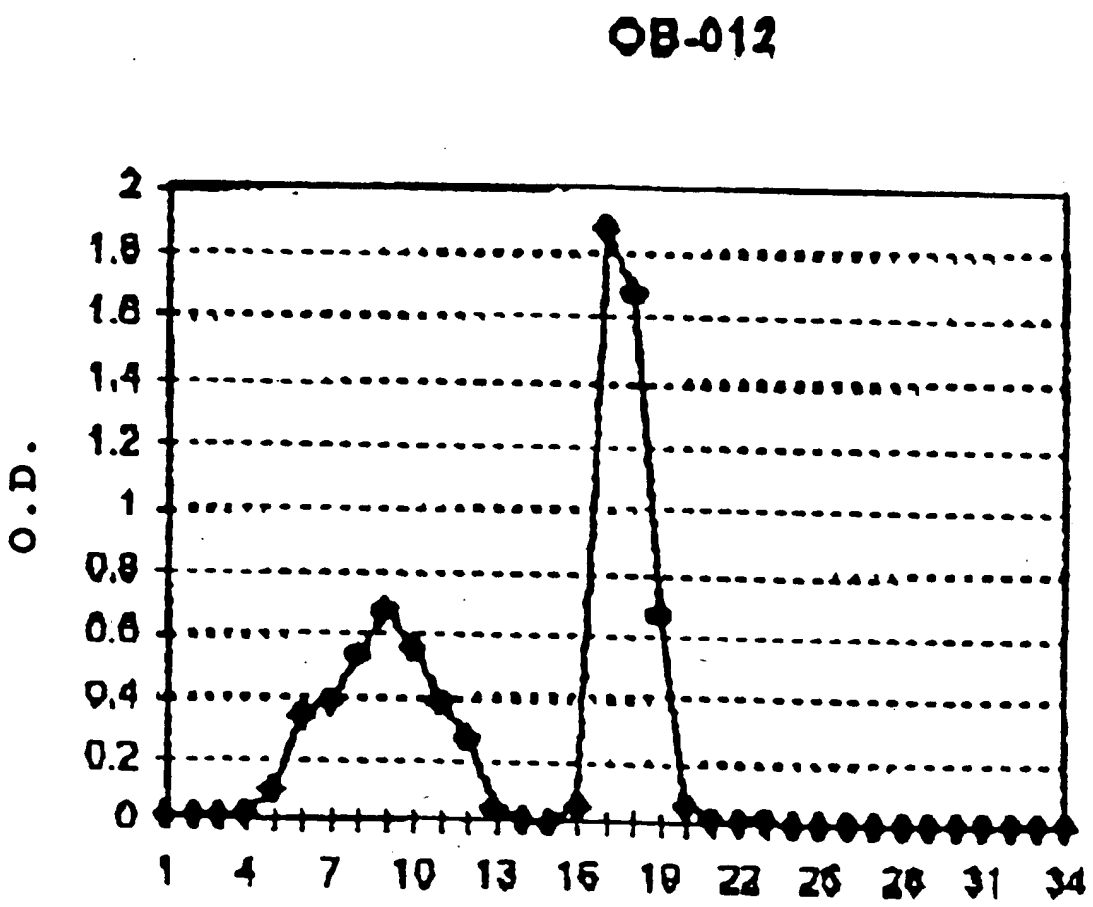
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Figure 2

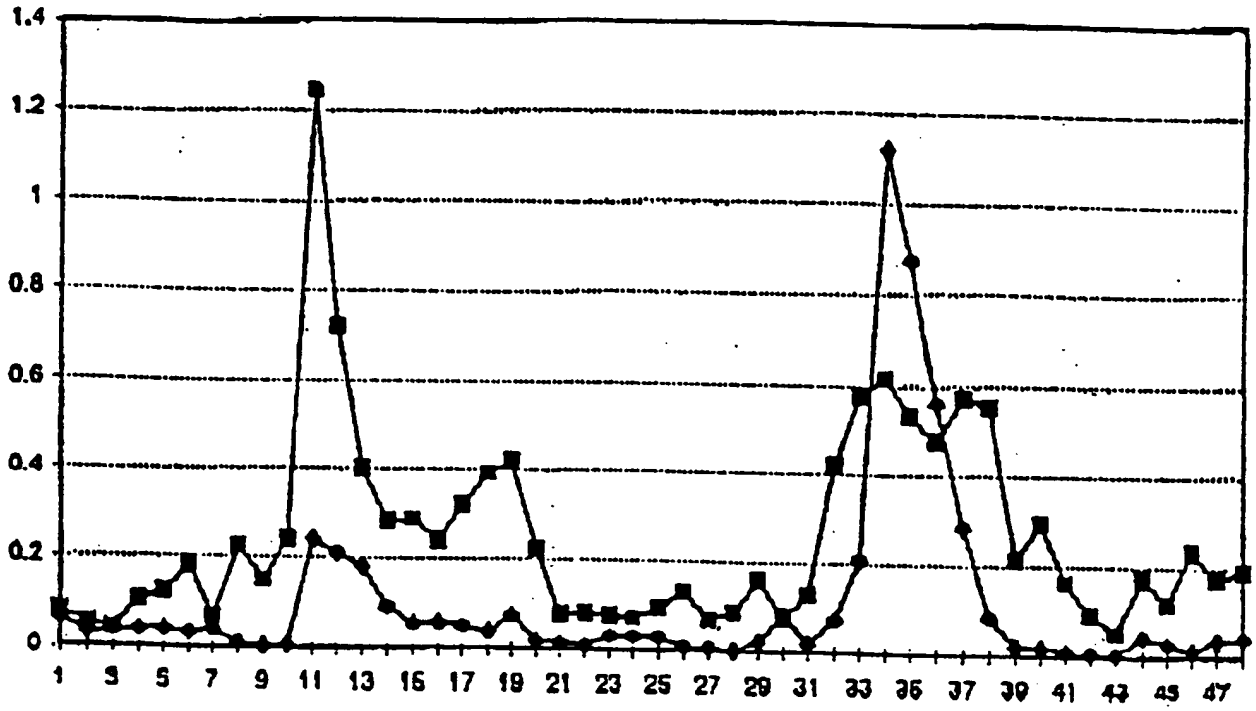
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Figure 3

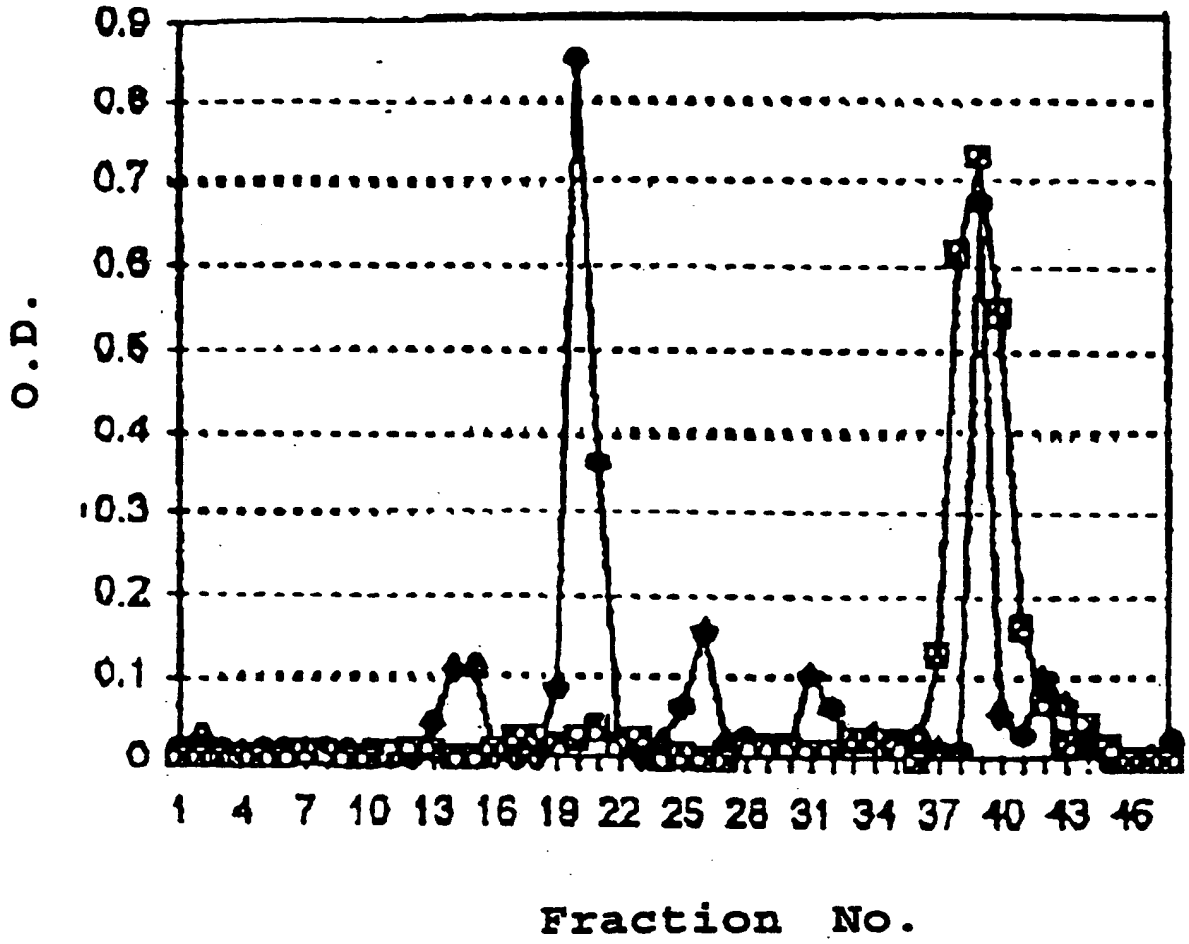
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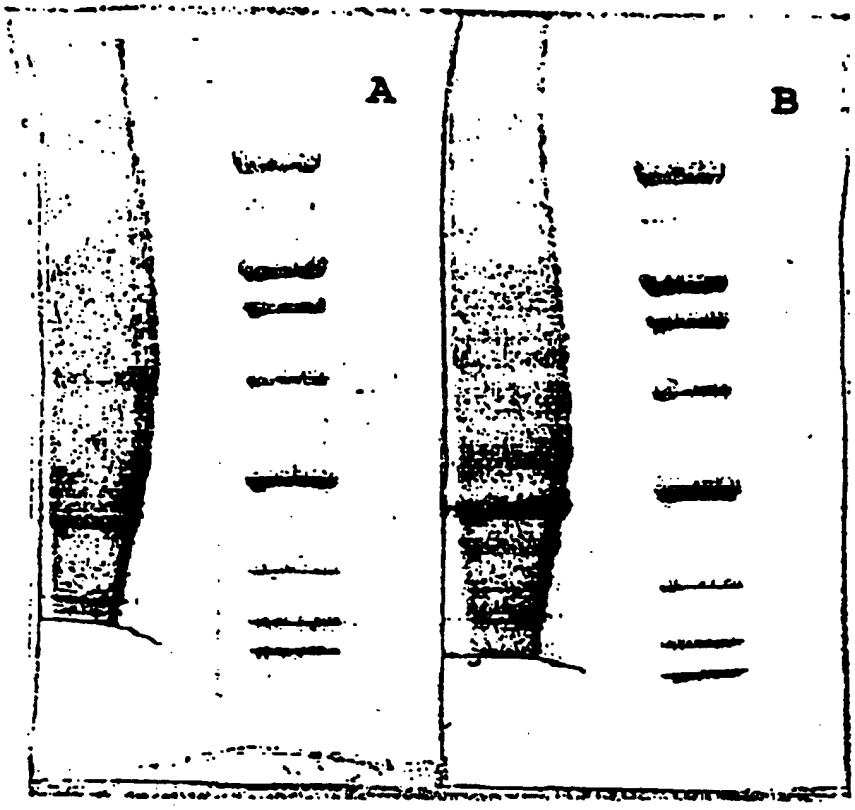
Figure 4



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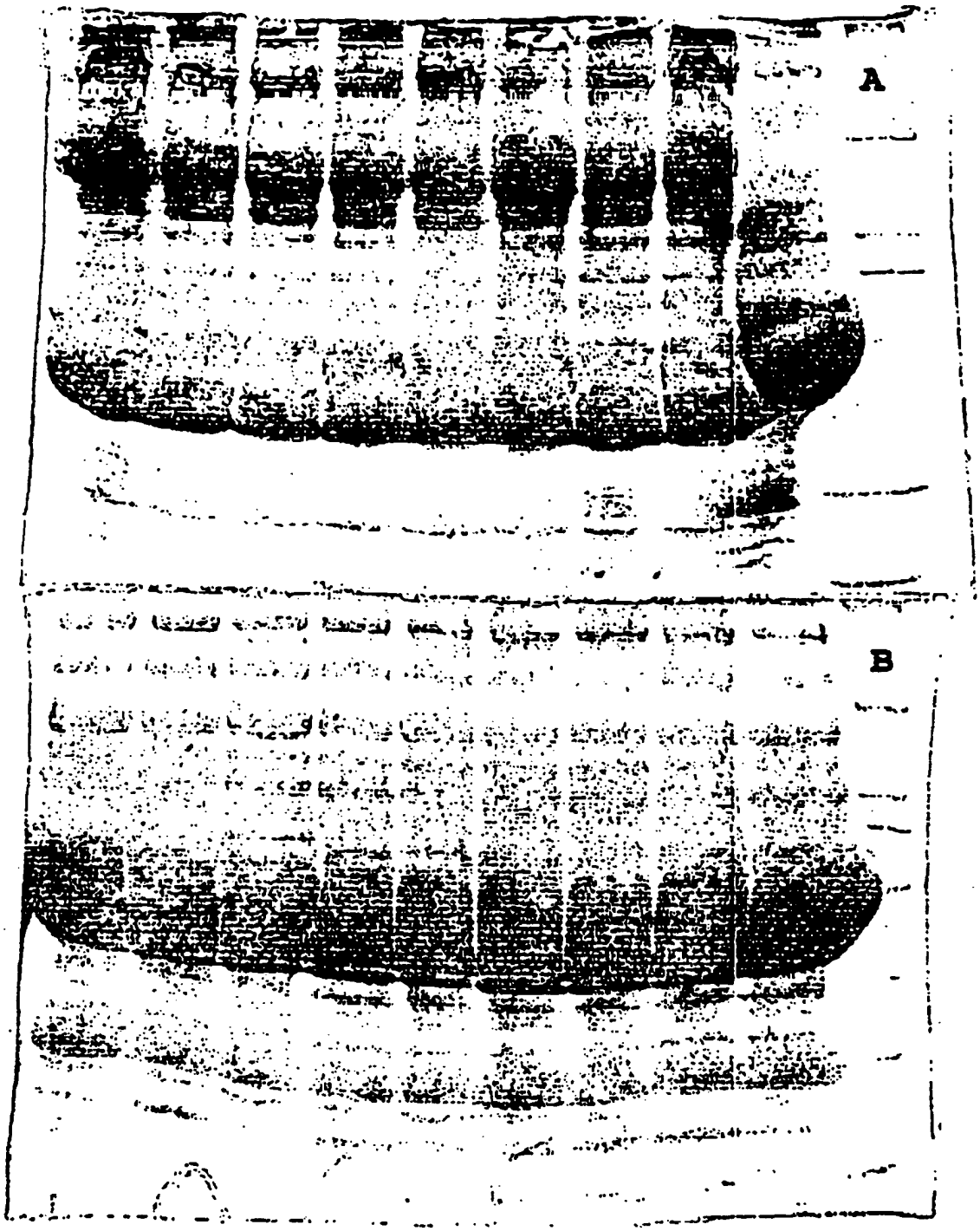

Figure 6

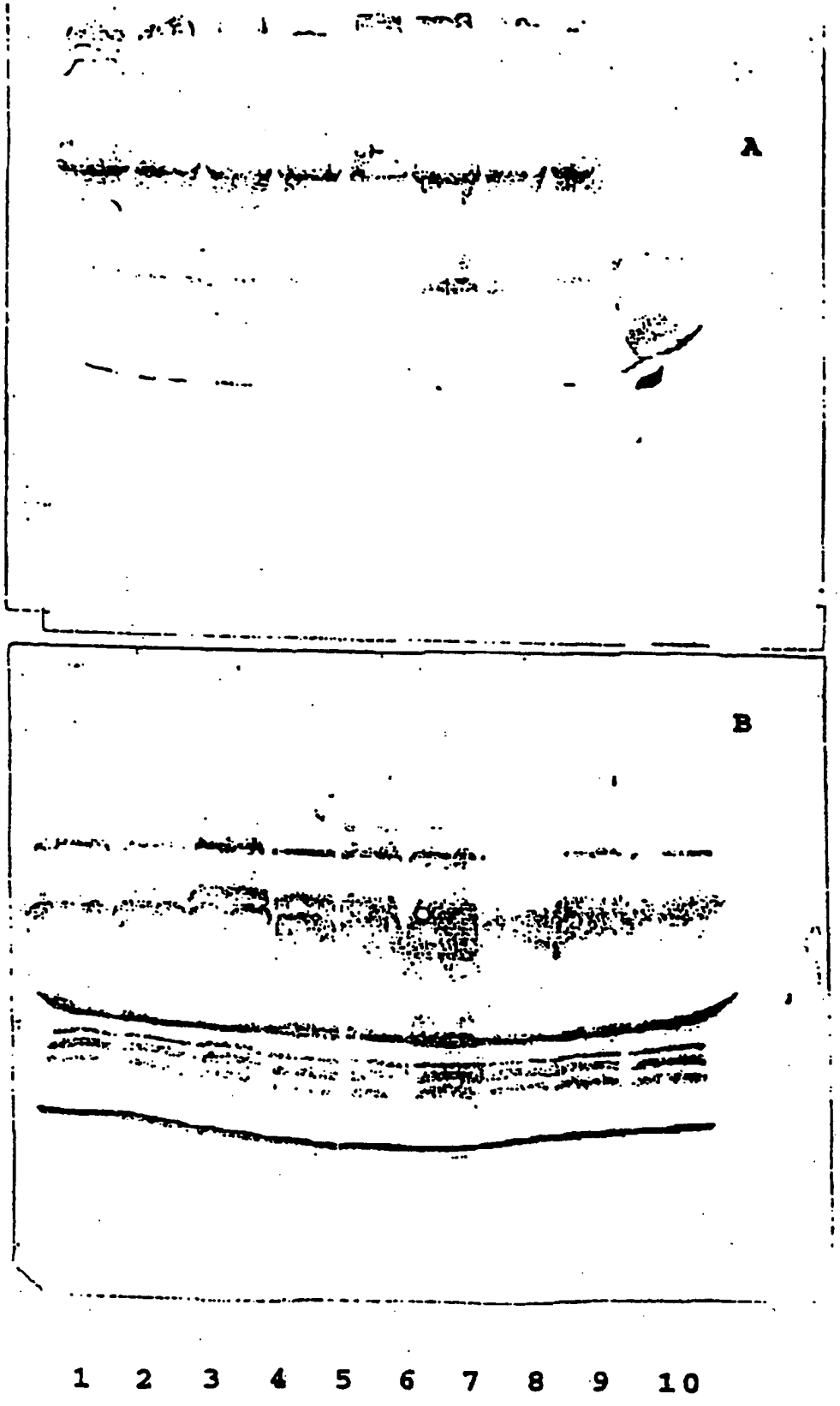

Figure 7

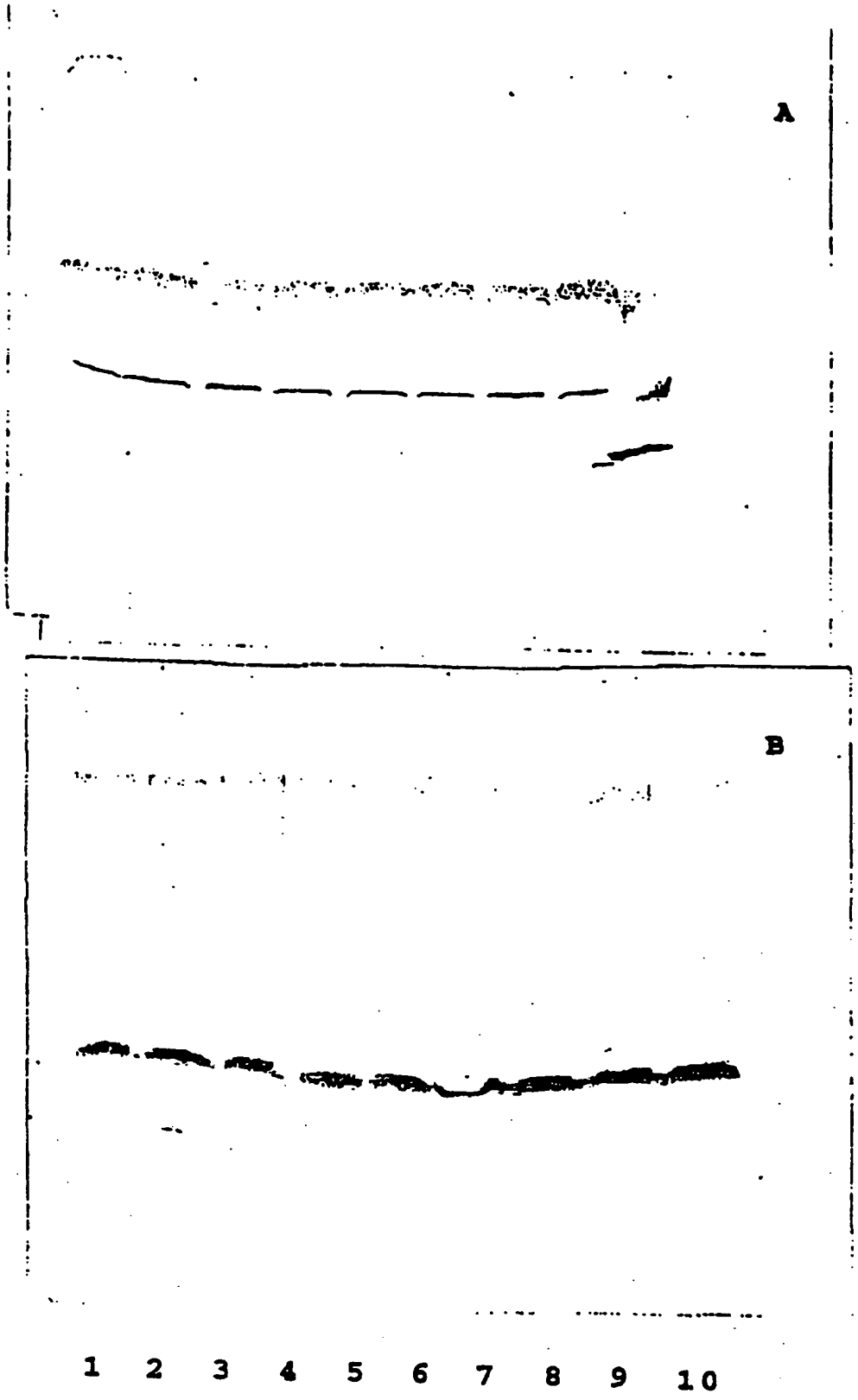

Figure 8

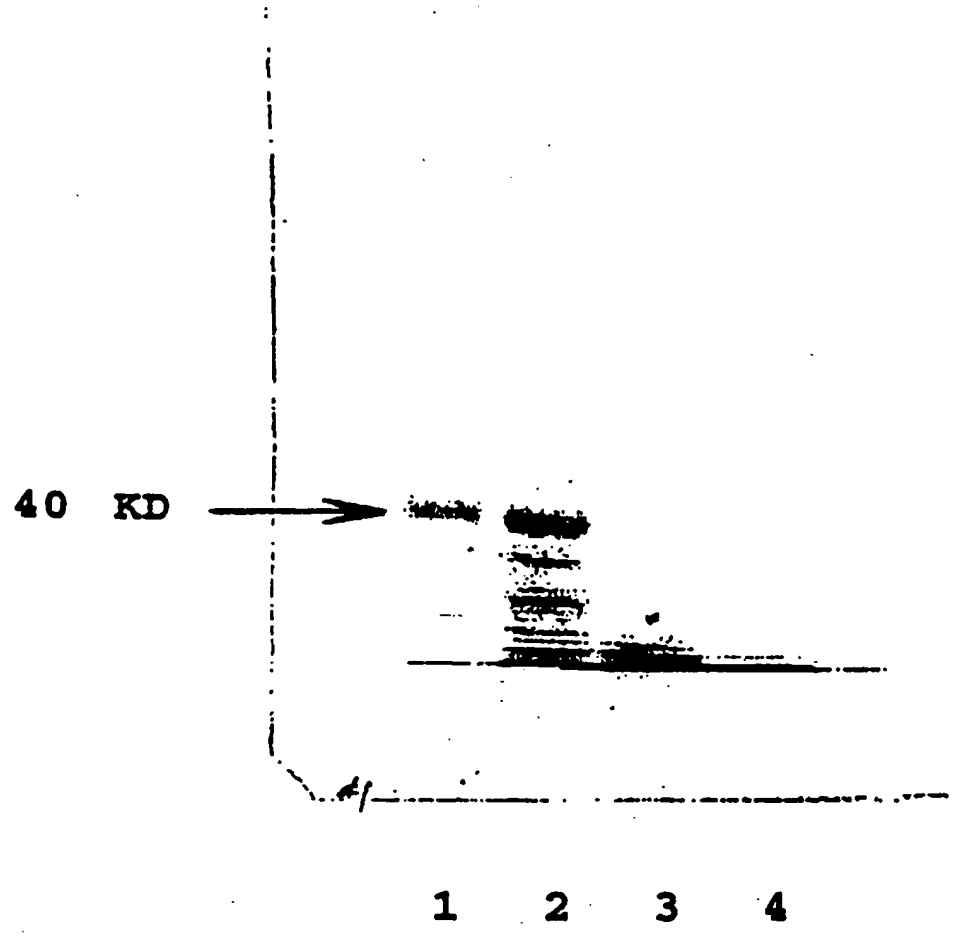

Figure 9

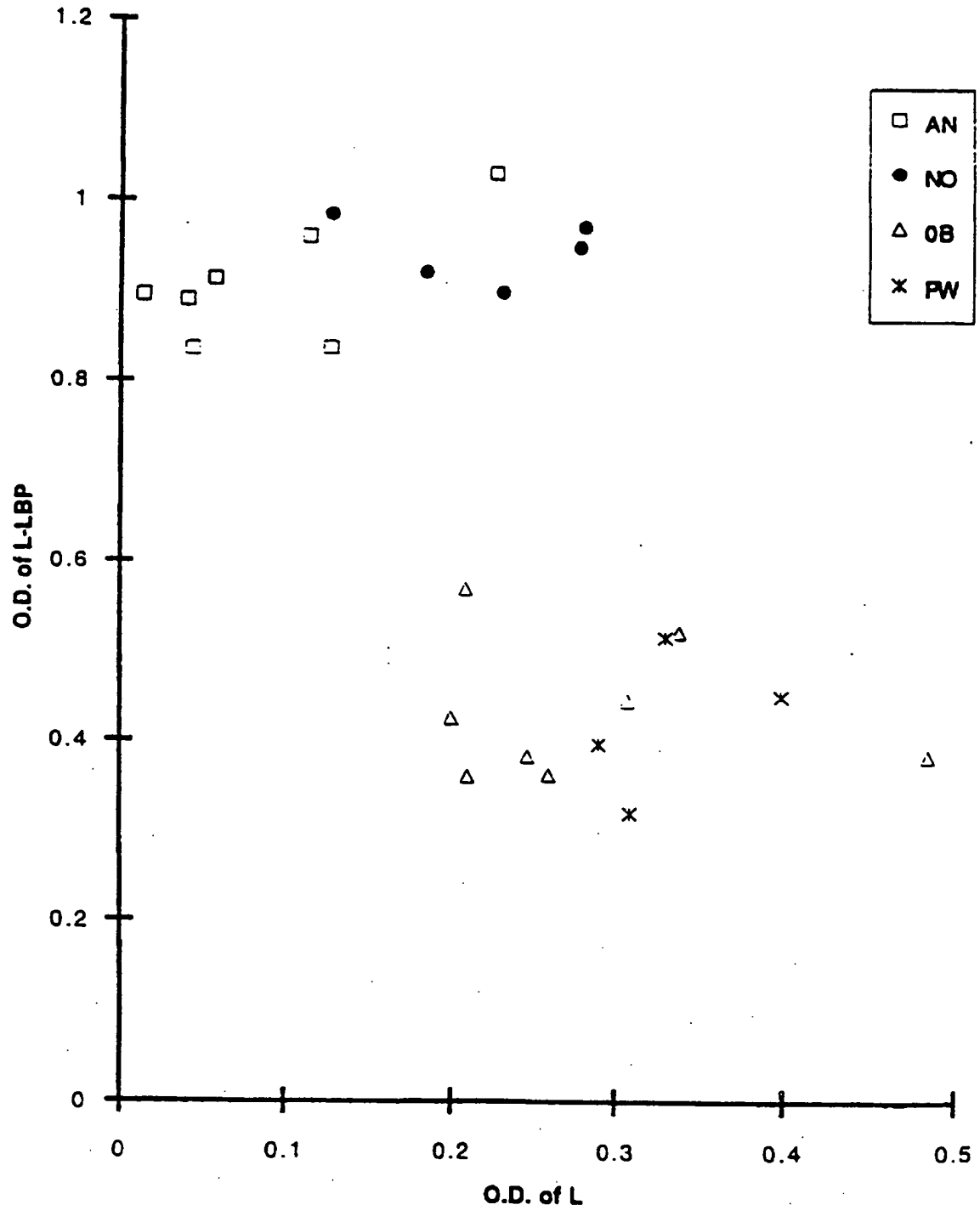

Figure 10A

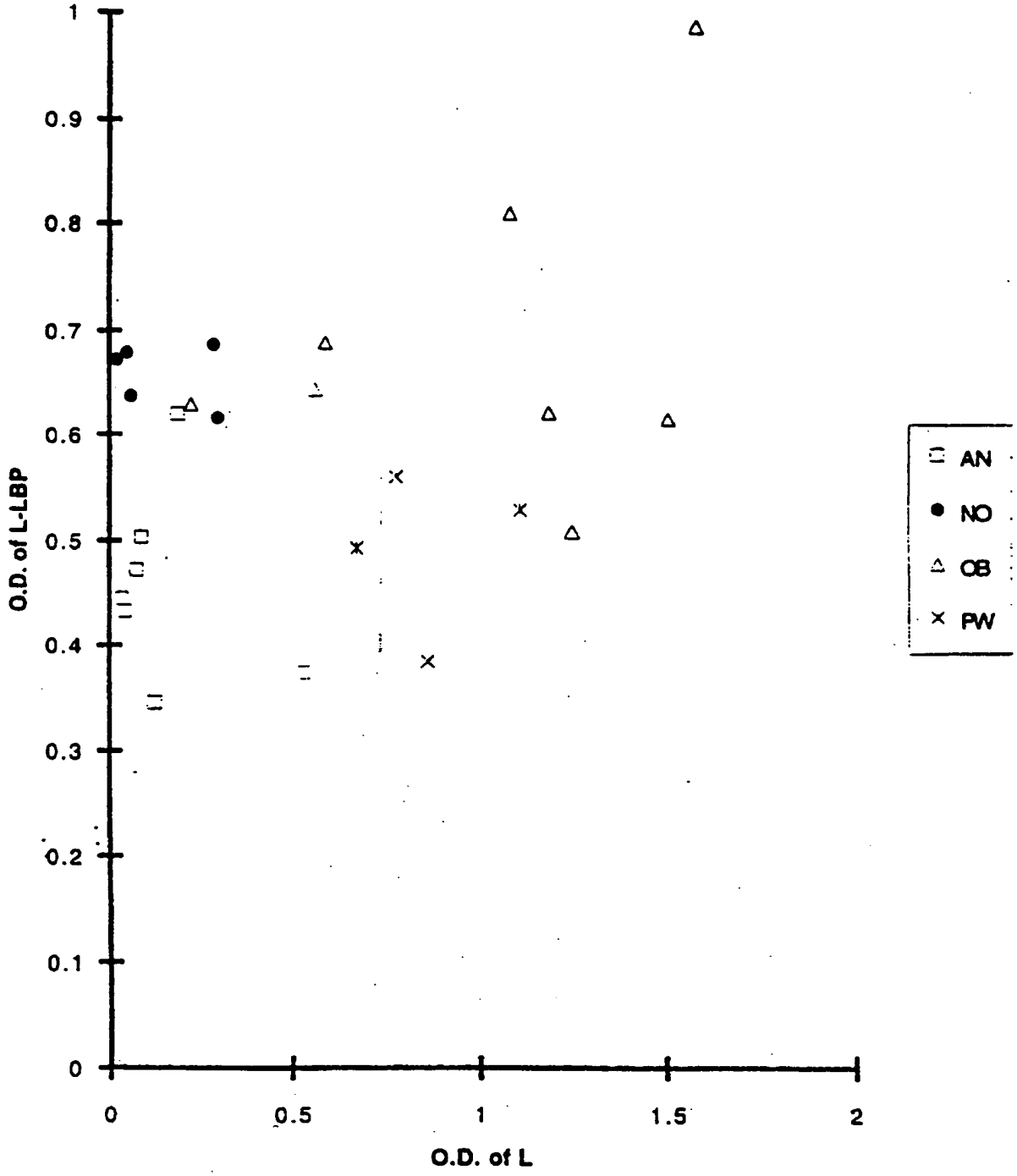

Figure 10B

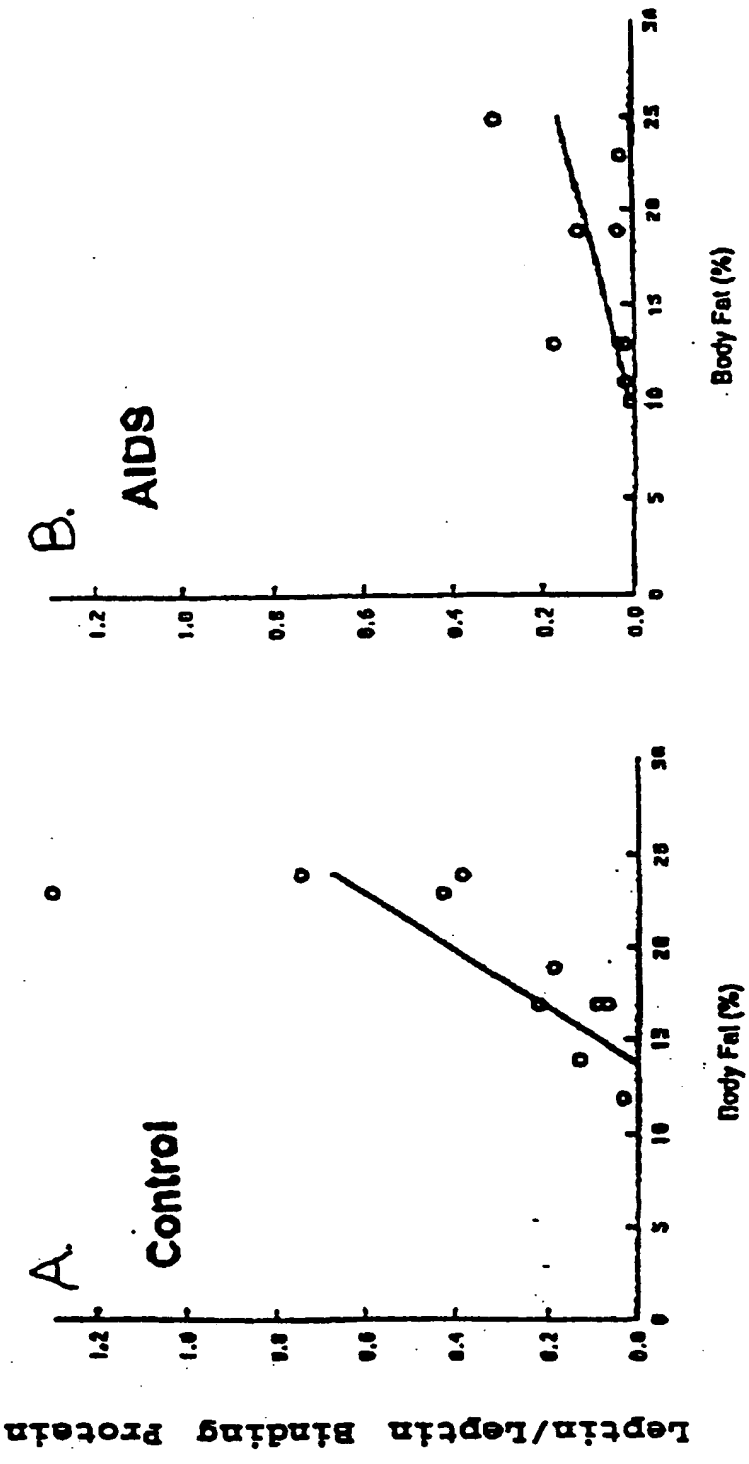
A.
Control
B.
AIDS
Leptin/Leptin Binding Protein
Body Fat (%)
Body Fat (%)

Figure 11

INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/US 97/14597

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/775 C07K14/575 C07K16/26 C07K16/18 A61K38/22
G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	M.K. SINHA ET AL.: "Evidence of Free and Bound Leptin in Human Circulation" JOURNAL OF CLINICAL INVESTIGATION, vol. 98, no. 6, 15 September 1996, pages 1277-1282, XP002047307 see page 1281, left-hand column, paragraph 3 - right-hand column, paragraph 4 ---	1,4-6,8
P,X	K.L. HOUSEKNECHT ET AL.: "Evidence for Leptin Binding to Proteins in Serum of Rodents and Humans: Modulation with Obesity" DIABETES, vol. 45, no. 11, November 1996, pages 1638-1643, XP002047308 see page 1641, right-hand column, paragraph 2 - page 1642, right-hand column, last paragraph --- -/--	1,4-6,8

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search
19 November 1997

Date of mailing of the international search report
12. 12. 97

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Authorized officer
Fuhr, C

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern .ial Application No
PCT/US 97/14597

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LEE G -H ET AL: "ABNORMAL SPLICING OF THE LEPTIN RECEPTOR IN DIABETIC MICE" NATURE, vol. 379, 15 February 1996, pages 632-635, XP002030818 see page 635, left-hand column, paragraph 1 ---	1,4-6,8
P,X	WO 97 26335 A (UNIV ROCKEFELLER) 24 July 1997 see page 6, paragraph 3 - page 7, paragraph 1 see page 13, paragraph 3 see page 17, paragraph 5 - page 18, paragraph 1; claims; examples ---	1,4-6,8
A	D.E. JENNE AND J. TSCHOPP: "Molecular structure and functional characterization of a human complement cytolysis inhibitor found in bloood and seminal plasma: Identity to sulfated glycoprotein 2, a constituent of rat testis fluid" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 86, September 1989, WASHINGTON US, pages 7123-7127, XP002047467 see page 7127, left-hand column, paragraph 2; figure 2 ---	2,3,13
A	WO 91 05043 A (SCHERING AG) 18 April 1991 see claims; examples -----	2,3,13

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/14597

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : As far as claims 8 and 17
are directed to a method of
treatment of the human/animal body or a method of diagnosis on the
human/animal body, the search has been carried out and based on the
alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern. ..al Application No
PCT/US 97/14597

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9726335 A	24-07-97	AU 2244797 A	11-08-97
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